Diversity and ontogeny of *Cryptococcus neoformans* var. *grubii* originating from South Africa

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not been previously in its entirety or in part been submitted at any university for a degree.

_____	1 December 2007
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SUMMARY

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic fungal pathogen responsible for causing meningitis predominantly in immuno-compromised individuals, particularly in those suffering from human immuno virus (HIV) acquired immuno-deficiency syndrome (AIDS). Two main varieties are known, *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A), as well as a hybrid variety, *C. neoformans* (serotype AD). These serotypes may belong to one of two mating types, namely mating type *a* (MAT*a*), or mating type alpha (MATα). Several molecular typing methods were previously developed to classify *C. neoformans* into four major genotypic groups, namely VNI, VNII, VNIII and VNIV. In addition to clinical samples, these yeasts are known to occur in a wide diversity of environmental habitats including soil, avian guano, trees and decaying wood.

The study had two main objectives, firstly to obtain an indication of the prevailing *C. neoformans* genotypes occurring within the HIV positive and AIDS populations of South Africa and to obtain an indication of its distribution within the environment, particularly within the Western Cape Province, South Africa. Secondly, to examine whether *C. neoformans* is able to grow and interact with selected microbes when cultured on woody debris and to determine if *C. neoformans* is capable of producing its ontogenic stages on this woody debris.

Despite attempts to isolate *C. neoformans* from 256 environmental samples originating from a variety of habitats in South Africa, a total of only four isolates were obtained from the environment. None were isolated from environmental sources in the Western Cape Province, South Africa. The four environmental *C. neoformans* var. *grubii* strains isolated from soil in the North West province of South Africa, and 32 clinical *C. neoformans* strains originating from the Gauteng and Western Cape provinces of South Africa were subsequently identified and characterized. Strains were identified by sequencing the internal transcribed spacer (ITS) region of the ribosomal gene cluster, while serotypes and mating types were confirmed using polymerase chain reaction (PCR) primers. The genotype of each strain was determined by employing three PCR-based

typing techniques, namely PCR fingerprinting using the mini-satellite M13, micro-satellite $(GACA)_4$ and random amplified polymorphic DNA (RAPD) analysis, as well as restriction fragment length polymorphism (RFLP) analysis of the phopholipase B1 gene. A total of 97 % of the strains were identified as *C. neoformans* var. *grubii* (serotype A), while only one strain was identified as *C. neoformans* var. *neoformans* (serotype D). All strains were found to be MATα and haploid. The majority of strains grouped into genotype VNI (75.6 %), seven strains represented genotype VNII (21.2 %), while only one strain represented genotype VNIV (3 %). These results are in accordance with previous and current literature stating that *C. neoformans* var. *grubii* (serotype A, MATα, VNI) is responsible for the majority of cryptococcal infections.

Using plate assays, all the *C. neoformans* strains were screened for wood degrading enzymes. All strains tested positive for cellulase activity, 6 % of strains tested positive for laccase production at 22 ºC, but no strains were able to degrade xylan. Subsequently, three *C. neoformans* var. *grubii* strains, originating from clinical and environmental samples, all representing the same genotype (VNI) and mating type (MATα), were evaluated for growth on *Acacia mearnsii* and *Eucalyptus camaldulensis* debris. While minimal differences were noted between strains, those cultured on *A. mearnsii* yielded significantly higher cell counts. Finally, all strains were mated on *Acacia mearnsii* and *Eucalyptus camaldulensis* debris, as well as V8 juice and yeast carbon base (YCB) agar to determine whether *C. neoformans* strains were capable of both dikaryotic and monokaryotic fruiting when cultured on woody debris. A total of 19 %, 6 %, 42 % and 72 % of the *C. neoformans* strains were able to mate when crossed on *A. mearnsii* and *E. camaldulensis* debris, V8 juice and YCB agar, respectively. Monokaryotic fruiting was observed in 3 %, 3 % and 3 % of strains when *C. neoformans* was cultured on *Acacia mearnsii*, *Eucalyptus camaldulensis* debris and YCB, respectively. This may be the first observation of *C. neoformans* in a hyphal phase when cultured on medium comprised solely of woody debris, the perceived natural habitat of this yeast.

OPSOMMING

Cryptococcus neoformans (Sanfelice) Vuillemin is 'n opportunistiese fungus patogeen wat verantwoordelik is vir breinvliesontsteking hoofsaaklik in immuno-gekompromiteerde individue, veral in diegene met menslike immunovirus (MIV) verworwe immuniteitsgebreksindroom (VIGS). Twee hoof variëteite is bekend, *C. neoformans* var. *neoformans* (serotipe D) en *C. neoformans* var. *grubii* (serotipe A), asook 'n hibried, *C. neoformans* (serotipe AD). Hierdie serotipes mag aan een van twee paringstipes behoort, naamlik paringstipe *a* (MAT*a*), of paringstipe alfa (MATα). Verskeie molekulêre tiperingsmetodes is voorheen ontwikkel om *C. neoformans* in vier hoof genotipiese groepe, naamlik VNI, VNII, VNIII en VNIV te klassifiseer. Benewens kliniese monsters, is hierdie giste bekend vir hul voorkoms in 'n wye verskeidenheid omgewingshabitatte, insluitende grond, voëlmis, bome en verrottende hout.

Die studie het twee hoof doelwitte, eerstens om 'n aanduiding te kry van die algemene *C. neoformans* genotipes wat in die MIV positiewe en VIGS populasies van Suid-Afrika voorkom asook die verspreiding daarvan in die omgewing, veral in die Wes-Kaap Provinsie. Tweedens, om te bepaal of *C. neoformans* in staat is om te groei en 'n interaksie met geselekteerde mikrobes te hê wanneer dit op houtagtige plantafval gekweek word en of *C. neoformans* in staat is om sy ontogeniese stadia op die plantafval te produseer.

Ten spyte van pogings om *C. neoformans* uit 256 omgewingsmonsters vanuit 'n verskeidenheid habitatte in Suid-Afrika te isoleer, is 'n totaal van slegs vier isolate uit die omgewing verkry. Nie een is uit omgewingsbronne in die Wes-Kaap van Suid-Afrika geïsoleer nie. Die vier omgewings *C. neoformans* var. *grubii* stamme, geïsoleer uit grond van die Noordwes Provinsie van Suid-Afrika, en 32 kliniese *C. neoformans* stamme afkomstig van die Gauteng en Wes-Kaap provinsies van Suid-Afrika, is vervolgens geïdentifiseer en gekarakteriseer. Stamme is geïdentifiseer deur die volgordebepaling van die interne getranskribeerde spasie (ITS) area van die ribosomale geengroep, terwyl serotipes en paringstipes is deur polimerase kettingreaksie (PCR) peilers bevestig. Die genotipe van elke stam is bepaal deur gebruik te maak van drie PCR-gebasseerde

tiperingstegnieke, naamlik PCR-tipering met behulp van die mini-satelliet M13, mikro-satelliet $(GACA)_4$ en lukraak ge-amplifiseerde polimorfiese DNA (RAPD) analise, asook beperkings-fragment-lengte-polimorfisme (RFLP) analise van die fosfolipase B1 geen. 'n Totaal van 97 % van die stamme is geïdentifiseer as *C. neoformans* var. *grubii* (serotipe A), terwyl slegs een stam as *C. neoformans* var. *neoformans* (serotipe D) geïdentifiseer is. Alle stamme was MATα en haploïed. Die meerderheid van die stamme is in genotipe VNI (75.6 %) gegroepeer, sewe stamme behoort tot genotipe VNII (21.2 %), terwyl net een stam genotipe VNIV (3 %) verteenwoordig. Hierdie resultate is in ooreenstemming met vorige en huidige literatuur wat aandui dat *C. neoformans* var. *grubii* (serotipe A, MATα, VNI) vir die meerderheid van cryptococcus-infeksies verantwoordelik is.

Al die *C. neoformans* stamme is vir houtdegraderende ensieme getoets deur middel van plaat-toetse. Alle stamme het positief getoets vir sellulase aktiwiteit, 6 % van die stamme het positief getoets vir lakkaseproduksie by 22 ºC, maar geen stamme was in staat om xilaan af te breek nie. Gevolglik is drie *C. neoformans* var. *grubii* stamme afkomstig van kliniese en omgewingsmonsters, almal verteenwoordigend van dieselfde genotipe (VNI) en paringstipe (MATα), geëvalueer vir groei op *Acacia mearnsii* en *Eucalyptus camaldulensis* afval. Terwyl minimale verskille tussen die twee stamme opgemerk is, het dié wat op *A. mearnsii* gekweek is, beduidend hoër selgetalle gelewer. Laastens is alle stamme op *A. mearnsii* en *E. camaldulensis* afval afgepaar, asook op V8 sap en gis-koolstofbasis (YCB) agar om te bepaal of *C. neoformans* stamme in staat is tot beide dikariotiese en monokariotiese vrugvorming wanneer dit op houtagtige afval gekweek word. 'n Totaal van 19 %, 6 %, 42 % en 72 % van die *C. neoformans* stamme was in staat om op onderskeidelik *A. mearnsii* en *E. camaldulensis* afval, V8 sap en YCB agar te paar. Monokariotiese vrugvorming is opgemerk in 3 %, 3 % en 3 % van die stamme wanneer *C. neoformans* op onderskeidelik *A. mearnsii*, *E. camaldulensis* afval en YCB gekweek is. Hierdie mag die eerste waarneming wees van *C. neoformans* in 'n hifeuse fase wanneer gekweek op 'n medium wat slegs uit houtagtige afval bestaan, die veronderstelde natuurlike habitat van hierdie gis.

MOTIVATION

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic fungal pathogen responsible for causing meningitis predominantly in immuno-compromised individuals (Casadevall *et al*., 2003; Franzot *et al*., 1998; Mitchell and Perfect, 1995), particularly those suffering from human immuno virus (HIV) acquired immuno-deficiency syndrome (AIDS). The incidence of this infection, also known as cryptococcosis, among these individuals is estimated at approximately 10 % (Chuck and Sande, 1989). Although the incidence of infection is less in organ transplant patients (5 %), the mortality rates are estimated at approximately 50 % of all cases (Vilchez *et al*., 2003; Husain *et al*., 2001).

This basidiomycetous yeast (Boekhout *et al*., 1997) is usually characterized by the production of melanin, resulting in typical brown colony pigmentation when cultured on differential media, particularly Niger seed (*Guizotia abyssinica*) agar (Yarrow, 1998). Two main varieties are known, *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A), with a hybrid variety, *C. neoformans* (serotype AD) also identified (Boekhout *et al*., 2001). Several molecular typing methods were developed to classify *C. neoformans* into four major genotypic groups, namely VNI, VNII, VNIII and VNIV (Latouche *et al*., 2003; Meyer *et al*., 1999).

C. neoformans is well known for its worldwide distribution (Sorrell *et al*., 1997) and has been isolated from a number of environmental sources including soil, avian guano contaminated soil, avian guano, fur trees, almond tress, eucalyptus trees, woody debris and decaying wood (Trilles *et al*., 2003; Halliday *et al*., 1999; Sorrell *et al*., 1997; Lazéra *et al*., 1996). It is clear however, that the variant *C. neoformans* var. *grubii* is more predominant with regards to environmental and clinical sources than *C. neoformans* var. *neoformans* with approximately 90 % of all clinical cases being attributed to this serotype (Mitchell and Perfect, 1995). As a result *C. neoformans* var. *grubii* is regarded as being more virulent than its counterpart *C. neoformans* var. *neoformans*.

Studies have shown that within sub-Saharan Africa, up to 30 % of AIDS patients suffer from cryptococcosis (Powderly, 1993). A recent survey conducted within the

Gauteng Province, South Africa, by the Gauteng Cryptococcal Surveillance Group during 2002 and 2003, were able to identify 1195 cryptococcosis cases during the first year (McCarthy *et al*., 2003). Cryptococcal meningitis accounted for 95 % of the cases, and the survey determined a mortality rate of 31 %. These relatively high mortality rates, despite antifungal treatments, emphasize the need to further examine this ubiquitous yeast pathogen to determine is true ecological niche in order to limit exposure and perhaps prevent infection of an increasing vulnerable HIV and AIDS population.

With the above as background the first objective of this study was to obtain an indication of the prevailing *C. neoformans* genotypes occurring within the HIV positive and AIDS populations of South Africa, and to obtain an indication of its distribution within the environment, particularly within the Western Cape Province (Chapter 2). The second objective of this study was to test whether clinical and environmental isolates of *C. neoformans* var. *grubii* are capable of growth and interaction with selected microbes when cultured on woody debris, the perceived natural habitat of this yeast (Chapter 3). Another goal was to test the hypothesis that *C. neoformans* is capable of producing all its ontogenic stages on woody debris.

LITERATURE CITED

Boekhout T., van Belkum A., Leenders A. C. A. P., Verbrugh H., Mukamurangwa P., Swinne D., Scheffers W. A. (1997). Molecular Typing of *Cryptococcus neoformans*: Taxonomic and Epidemiological Aspects. *Int. J. Sys. Bacteriol.* **47, 2:** 432-442.

Boekhout T., Theelen B., Diaz M., Fell J. W., Hop W. C. J., Abeln E. C. A., Dromer F., Meyer W. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiol.* **147:** 891-907.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready made' virulence and 'dual use' virulence factors in pathogenic environment – the *Cryptococcus neoformans* paradigm. *Curr. Opin. Microbiol.* **6:** 332-337.

Chuck S. L., and Sande M. A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **321:** 794–799.

Franzot S. P., Fries B. C., Cleare W., Casadevall A. (1998). Genetic Relationship between *Cryptococcus neoformans* var. *neoformans* strains of serotypes A and D. *J. Clin. Microbiol*. **36, 8:** 2200-2204.

Halliday C., Bui T., Krockenberger M., Malik R., Ellis D., Carter D. (1999). Presence of α and a mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gattii* strains from Australia. *J. Clin. Microbiol.* **37:** 2920-2926.

Husain S., Wagener M. M., Singh N. (2001). *Cryptococcus neoformans* infection in organ transplant recipients: variables influencing clinical characteristics and outcome. *Emerg. Infect. Dis.* **7:** 375-381.

Latouche G. N., Huynh M., Sorrell T. C., Meyer W. (2003). PCR-Restriction fragment length polymorphism analysis of the Phospholipase B (*PLB1*) gene for sub-typing of *Cryptococcus neoformans* isolates. *Appl. Environ. Microbiol*. **69:** 2080-2086.

Lazéra M. S., Pires F. D., Camillo-Coura L., Nishikawa M. M., Bezerra C. C., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *J. Med. Vet. Mycol.* **34, 2:** 127-31.

McCarthy K., Crewe-Borwn H. H., Mhlongo N., Mirza S., Brandt M., Haijeh R and the Gauteng Cryptococcal Surveillance Group (2003). The Burden of Cryptococcosis in Gauteng Province (Johannesburg), South Africa: Results of Population-based Active Surveillance, 2002-2003. Communicable Diseases Surveillance Bulletin (http://www.nicd.ac.za), 10-12.

Meyer W., Marszewska K., Amimostofian M., Igreja R. P., Hardtke C., Methling K., Viviani M. A., Chindamporn A., Sukroongreung S., John M. A., Ellis D. H., Sorrell T. C. (1999). Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA - a pilot study to standardize techniques on which to base a detailed epidemiological survey. *Electrophoresis.* **20:** 1790-1799.

Mitchell T. G. and Perfect J. R. (1995). Cryptococcosis in the Era of AIDS—100 Years after the Discovery of *Cryptococcus neoformans*. *Clin. Microbiol. Rev.* **8, 4:** 515-548.

Powderly W. G. (1993). Cryptococcal meningitis and AIDS. *Clin. Infect. Dis.* **17:** 837–842.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia.* **151:** 63-69.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans*. *Rev. Iberoam. Microbiol*. **14:** 42-43.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Med. Mycol.* **41, 5:** 383-390.

Vilchez R., Shapiro R., McCurry K., Kormos R., Abu-Elmagd K., Fung J., Kusne S. (2003). Longitudinal study of cryptococcosis in adult solid-organ transplant recipients. *Transpl Int.* **16:** 336-340.

Yarrow D. (1998). Methods for isolation, maintenance and identification of yeasts. In: Kurtzman C. P., Fell J. W. (Eds.) *The Yeasts, a Taxonomic Study*. Elsevier Science BV, Amsterdam, pp. 77-98.

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“Grasp the subject,

the words will follow.”

- Cato the Elder

TABLE OF CONTENTS

CHAPTER 1

Literature Review

1. *CRYPTOCOCCUS NEOFORMANS*: THE YEAST 2

2. *CRYPTOCOCCUS NEOFORMANS*: THE PATHOGEN 3

3. SEXUAL REPRODUCTION OF *CRYPTOCOCCUS NEOFORMANS* 7

3.1. Dikaryotic Mating in C. neoformans 7

3.2. Monokaryotic Fruiting in C. neoformans 8

3.3. Genetic Composition of the MAT locus of C. neoformans 10

3.4. Pheromone Response Pathway of C. neoformans 10

3.5. The Link between Mating Type and Virulence 12

4. VIRULENCE FACTORS OF *CRYPTOCOCCUS NEOFORMANS* 13

4.1. The Polysaccharide Capsule 13

4.1.1. Polysaccharide Capsule Structure 14

4.1.2. Regulation of Capsule Biosynthesis 14

4.1.3. Role of the Polysaccharide Capsule during Pathogenesis of *C. neoformans* 15

4.2. Laccase 16

4.2.1. The Cryptococcal Laccase Enzyme 16

4.2.2. Molecular Regulation of the Cryptococcal Laccase Enzyme 17

4.2.3. Cellular Location of the Cryptococcal Laccase Enzyme 17

4.2.4. Role of the Laccase Enzyme during Pathogenesis of *C. neoformans* 18

4.3. Melanin 18

4.3.1. Biosynthesis of Melanin in *C. neoformans* 19

4.3.2. The Role of Melanin during Pathogenesis of *C. neoformans* 20
4.4. Thermo-tolerance 20
4.5. Additional virulence factors 21
4.5.1 Phospholipase 21
4.5.2. Urease 22
4.5.3. Proteinase 22

5. ORIGINS OF VIRULENCE IN *CRYPTOCOCCUS NEOFORMANS* 22
5.1. *Microbial Interactions* 22
5.2. Predation 23

6. FINDING THE TRUE ECOLOGICAL NICHE OF *CRYPTOCOCCUS NEOFORMANS* 24

7. CONCLUSIONS 25

8. PROJECT OBJECTIVES 26

9. LITERATURE CITED 27

CHAPTER 2

Isolation, Identification and Characterization of *C. neoformans* Strains Originating from Three Provinces of South Africa

1. ABSTRACT 43

2. INTRODUCTION 43

3. MATERIALS AND METHODS 46
3.1. Strains and culture conditions 46

3.2. Sampling 46
3.3. Isolation from environmental sources 46
3.4. Preliminary Identification 47
3.4.1. Growth on Differential Medium 47
3.5. Molecular Identification 47
3.5.1. Genomic DNA extraction 47
3.5.2. Analysis of the internal transcribed spacer (ITS) region 48
3.6. Molecular Characterization 48
3.6.1. Serotype determination using PCR specific primers 48
3.6.2. Mating type determination using PCR specific primers 49
3.6.3. Genotyping 49

4. RESULTS AND DISCUSSION 51
4.1. Isolation of *C. neoformans* from environmental sources 51
4.2. Preliminary identification 52
4.2.1. Growth on Differential Media 52
4.3. Molecular identification 52
4.4. Molecular Characterization 55
4.4.1. Serotype and mating type determination using PCR specific primers 55
4.4.2. Genotyping 57

5. CONCLUSIONS 57

6. LITERATURE CITED 62

CHAPTER 3

Interactions of *C. neoformans* var. *grubii* within a woody environment

1. ABSTRACT 71

2. INTRODUCTION 71

3. MATERIALS AND METHODS 74

3.1. Strains and culture conditions 74

3.2. Screening for wood degrading enzymes 75

3.2.1. Laccase activity 75

3.2.2. Cellulase activity 75

3.2.3. Xylanase activity 76

3.3. Testing for the growth of *C. neoformans* var. *grubii* on woody debris 76

3.3.1. Preparation of the woody debris 76

3.3.2. Physico-chemical analysis of the woody debris 76

3.3.3. Preparation of woody debris solid state cultures containing C. neoformans *var*. grubii 77

3.4. Survival of *C. neoformans* var. *grubii* in woody debris in the presence of selected microbes 78

3.4.1. Enrichment for Protista 78

3.4.2. Preparation of Sterile Soil Solution and C. neoformans *var*. grubii *cultures* 79

3.4.3. Preparation of woody substrate solid state cultures containing C. neoformans *var.* grubii *and protista* 79

3.4.4. Isolation and identification of bacteria 80

3.4.5. Preparation of woody substrate solid state cultures containing Pseudomonas fluorescens *or* Enterobacter sp. 81

3.4.6. Preparation of woody substrate solid state cultures containing

C. neoformans *var.* grubii *and* Pseudomonas fluorescens *or* Enterobacter sp. 82

3.4.7. Preparation of woody substrate solid state microcosms containing C. neoformans *var.* grubii, P. fluorescens, Enterobacter *sp as well as predatory protists* 82

3.5. Fruiting of *C. neoformans* on woody debris 83

3.6. Fruiting of *C. neoformans* on standard media 83

3.6.1. Fruiting of C. neoformans *on V8 juice agar* 83

3.6.2. Fruiting of C. neoformans *on nitrogen limited media – Yeast Nitrogen Base* 83

4. RESULTS AND DISCUSSION 84

4.1. Screening for wood degrading enzymes 84

4.1.1. Laccase activity 84

4.1.2. Cellulase activity 84

4.1.3. Xylanase activity 86

4.2. Testing for the growth of *C. neoformans* var. *grubii* on woody debris 87

4.2.1. Physico-chemical analysis of the woody debris 87

4.2.2. Survival of C. neoformans *on woody substrate* 89

4.3. Survival of *C. neoformans* var. *grubii* in woody debris in the presence of selected microbes 93

4.4. The ability of *C. neoformans* to fruit on woody debris 96

4.5. Fruiting of *C. neoformans* on standard media 101

5. CONCLUSIONS 104

6. LITERATURE CITED 105

CHAPTER 4

General Conclusions and Future Research

1. GENERAL CONCLUSIONS AND FURTURE RESEARCH 115

2. UNANSWERED QUESTIONS 117

3. LITERAURE CITED 117

APPENDICES

APPENDIX A 121

APPENDIX B 124

APPENDIX C 127

APPENDIX D 139

Chapter 1

Literature Review

1. *CRYPTOCOCCUS NEOFORMANS*: THE YEAST

Cryptococcus neoformans (Sanfelice) Vuillemin, anamorph of *Filobasidella neoformans*, is a facultative intracellular opportunistic pathogen causing cryptococcosis in immuno-suppressed individuals, such as those suffering from acquired immuno-deficiency syndrome (AIDS) cancer, and those receiving immuno-suppressive therapy (Casadevall *et al*., 2003; Harrison, 2000). It belongs to the order Tremellales, also called the jelly fungi, that is predominantly comprised of basidiomycetous yeasts (Boekhout *et al*., 2001; Fell *et al*., 2000). The yeast *C. neoformans* is encapsulated and can appear either round or oval shaped, is able to utilize a broad variety of carbon compounds as a growth substrate and is considered to be prototrophic for the majority of sugars, amino acids and lipids (Casadevall *et al*., 2003; Steenbergen *et al*., 2003). The ability to withstand physiological temperatures (37 °C) is characteristic of most pathogens; however *C. neoformans* is also able to tolerate a range of environmental temperatures and does not require a host cell in order to replicate. Yeast reproduction occurs both asexually and sexually (Kwon-Chung, 1980). While asexual reproduction occurs through budding of the yeast cell, sexual reproduction is due to the conjugation of yeast cells of the opposite mating types namely, mating type *a* (MAT*a*) and mating type α (MATα). The result is a dikaryotic mycelium that gives rise to basidia followed by meiosis and the production of haploid basidiospores.

Until recently there were thought to be three variants within the species complex each containing antigenic determinants that yield four serotypes, namely *C. neoformans* var. *neoformans* (serotype D); *C. neoformans* var. *grubii* (serotype A) and finally *C. neoformans* var. *gattii* (Sanfelice) Vuillemin (serotypes B and C). The emergence of a fifth serotype, serotype AD, a hybrid between *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* raised speculation over the grouping and classification of these three variants. Indeed a number of discrepancies had been previously noted between *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii* and *C. neoformans* var. *gattii* and included differences in their biochemistry, environmental distribution, DNA composition, chromosome numbers, as well their host preference. In 2001, Boekhout and co-workers resolved by means of amplified fragment length polymorphism (AFLP) techniques that *C. neoformans* var. *gattii* should be regarded as a separate species to *C. neoformans* var. *neoformans* and

C. neoformans var. *grubii* has since been re-classified as *Cryptococcus gattii*. These findings were confirmed by molecular evolutionary studies indicating that *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* have in fact been genetically separated for approximately 18 million years while *C. gattii* diverged from the lineage before that time period (Perfect, 2005).

2. *CRYPTOCOCCUS NEOFORMANS*: THE PATHOGEN

C. neoformans is the leading cause of fungal meningitis in immune impaired individuals world-wide and results in an inflammation of the meninges, the membranes that cover and protect the brain and spinal cord (Saag *et al*., 2000). The yeast was only recognized as a human pathogen in 1894 before the development of antimicrobial drugs, venous catheters, immuno-suppressive drugs and the increasing prevalence of the human immuno virus (HIV) and AIDS. Until then systemic fungal infections, such as cryptococcosis, were considered to be extremely rare (Casadevall, 2005; Steenbergen *et al*., 2003).

Today *C. neoformans* is responsible for approximately 6-10% of all AIDS-related infections and 5 % of infections seen in organ transplant recipients (Vilchez *et al*., 2003; Husain *et al*., 2001; Liu *et al*., 1999; Williamson, 1997; Wang *et al*., 1996; Chuck and Sande, 1989). Mortality rates are generally high, up to 50 % in organ transplant patients, despite the use of anti-fungal therapies to combat the yeast. Interestingly, while *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are routinely isolated from immune impaired individuals *C. gattii* has been reported in an ever increasing number of cases involving immune-competent individuals, particularly in British Columbia, Vancouver Island, Canada (Fraser *et al*., 2003; Taylor *et al*., 2002).

The exact mechanism of infection is not yet clearly understood. It is suspected that infectious propagules, such as desiccated yeast cells and aerosolized basidiospores, are inhaled (Fig 1) and may remain localized within the lungs (Feldmesser *et al*., 2001). Pulmonary cryptococcosis is generally asymptomatic although minor symptoms are often confused with those of viral infections (Goldman *et al*., 2001; Goldman *et al*., 2000). Upon dissemination the pathogen shows a preference for the central nervous system (CNS) resulting in meningitis, meningoencephalitis or expanding cryptococcoma (Feldmesser *et al*., 2001; Saag *et*

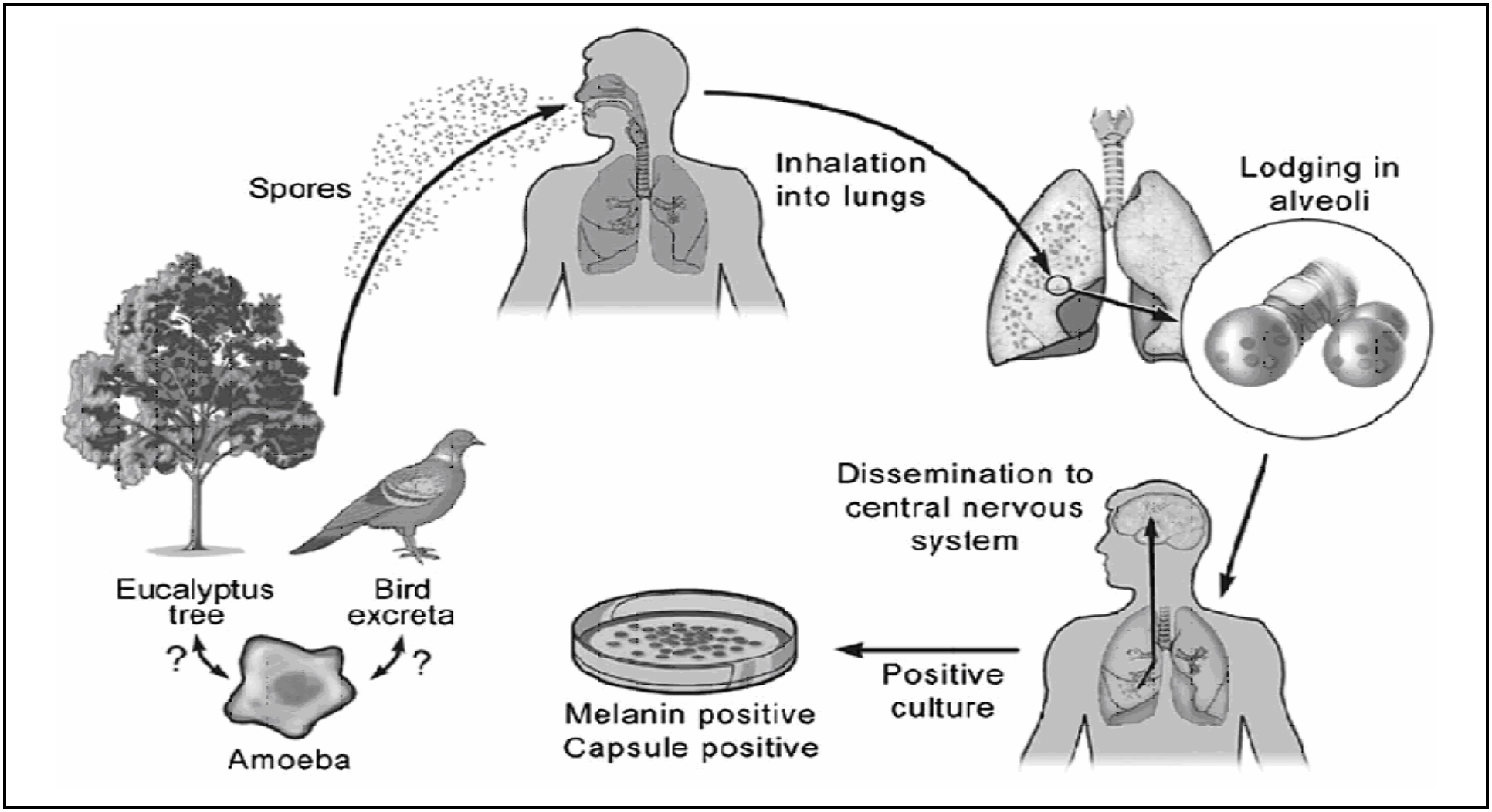

Figure 1 The proposed infection pathway of the pathogenic yeast *C. neoformans* (Hull and Heitman, 2002).

al., 2000). The mechanism by which the pathogen is able to traverse the blood-brain barrier has not yet been fully elucidated. Chrétien and co-workers (2002) were able to demonstrate that *C. neoformans* circulates throughout the body within blood monocytes and is also capable of entering endothelial cells of leptomeningeal capillaries highlighting a possible means of traversing the blood-brain barrier (Ibrahim *et al*., 1995).

The central nervous system however, is not the only bodily organ that may be affected. *Cryptococcus neoformans* is also known to infect the skin, causing cutaneous cryptococcosis that manifests in the form of lesions and ulcers, the bones and other visceral organs. Once again dissemination is believed to occur by means of infected macrophages and blood monocytes (Feldmesser *et al*., 2001; Saag *et al*., 2000).

The pathogen's mechanisms of *in vivo* survival within the macrophages, monocytes and endothelial cells remain unclear (Chrétien *et al*., 2002; Goldman *et al*., 2000). Studies have suggested that the body's immune system may not be able to fully eradicate the yeast, but simply compartmentalizes the infection. Goldman and co-workers were able to show that during persistent pulmonary infection in the rat, the pathogen was primarily located within the alveolar macrophages and the intracellular spaces of epithelioid cells (Goldman *et al*., 2000; Nessa *et al*., 1997). These persistent infections have been attributed to a number of virulence factors expressed by *C. neoformans* including capsule and melanin production (Steen *et al*., 2002; Zhu *et al*., 2001; Liu *et al*., 1999).

Interestingly, studies have revealed that exposure to *C. neoformans* occurs at an early age and that the majority of children two years and older acquire life long antibodies targeted towards the pathogen (Goldman *et al*., 2001). This suggests that childhood infections may remain relatively asymptomatic and that the yeast cells may lie dormant only to be "re-activated" later on in life once the immune system is compromised. Indeed studies have shown that *C. neoformans* infection may be latent for up to 18 years or more. Based on molecular profiling, random amplified polymorphic DNA (RAPD) and *C. neoformans* repetitive element 1 (CNRE-1) restriction fragment length polymorphism (RFLP), Garcia-Hermoso and co-workers (1999) were able to show that *C. neoformans* isolates from African patients differed from those isolates from French patients despite their lack of exposure towards African continent for an average of 13 years.

Until the onset of the 1950s, disseminated cryptococcosis was uniformly fatal (Saag *et al*., 2000). With the introduction of a number of anti-fungal agents, including amphotericin B (AMB), flucytosine, fluconazole and itraconazole, the successful treatment of cryptococcosis has improved. The introduction of AMB in the 1950s resulted in the successful treatment of up to 70 % of cases. AMB was replaced with flucytosine, an orally bio-available agent that demonstrated a potent activity against *C. neoformans*. However, the over-use of fluctysine led to the rapid development of fluctysine resistance in *C. neoformans*. Later research would also implicate the extended use of fluctysine in toxicity, particularly in patients with a compromised immune system (Viviani, 1996). Towards the early 1980s two new orally bio-available azole anti-fungal agents, namely fluconazole and itraconazole, were introduced. Both displayed activity against *C. neoformans*.

Treatment is primarily determined by the site of infection, such as pulmonary or meningitis, as well as the status of the patient's immune system (Saag *et al*., 2000). Both factors will influence the dosage of the anti-fungal agent as well as the length of treatment. The preferred treatment in HIV negative patients is determined largely by the site of infection, namely the lungs or central nervous system. Patients presenting pulmonary infections are usually treated with 200-400 mg/d fluconazole or itraconazole for six to 12 months (Pappas *et al*., 1998; Dromer *et al*., 1996; Denning *et al*., 1989). Those presenting a central nervous system infection are generally prescribed a combination of AMB and flucytosine for two weeks followed by 400 mg/d fluconazole for a minimum of 10 weeks (van der Horst *et al*., 1997).

Patients suffering with a compromised immune system, such as HIV and AIDS, are prescribed a primary therapy followed by extended therapy, termed maintenance therapy. Maintenance therapy involves the lifelong supplementation of the immune system with one or more anti-fungal agents. Patients presenting pulmonary infections are generally prescribed 200-400 mg/d fluconazole or itraconazole lifelong, or alternatively, 400 mg/d fluconazole supplemented with fluctysine for a minimum of 10 weeks (Jones *et al*, 1991; Denning *et al*., 1989). Those patients presenting central nervous system infections are generally treated with a combination of AMB and flucytosine for two weeks, followed by 400 mg/d fluconazole for a minimum of 10 weeks (van der Horst *et al*., 1997; Powderly, 1993). Fluconazole (200-400 mg/d) is generally prescribed during maintenance therapy as this azole is considered to be more effective, although itraconazole (200 mg/d) and AMB have also proven to be successful (Mondon *et al*., 1999; Saag *et al*., 1999; Powderly *et al*., 1992).

To date reports highlighting the emerging resistance of *C. neoformans* to anti-fungal agents are relatively limited. The long term use of fluconazole by AIDS patients however, is of concern with regards to the emergence of more resistant strains within the population. Totorano and co-workers (1993) examined the *in vitro* resistance of 153 *C. neoformans* strains to fluconazole and noted that 26 % were indeed resistant. A total of six fluconazole resistant strains were isolated from AIDS patients receiving fluconazole as maintenance therapy. Interestingly, a later study conducted by Brandt and co-workers (2001), noted no significant shift in the minimum inhibitory concentrations (MICs) of fluconazole over the period from 1992 to 1998. Despite the relative uncommon resistance seen in *C neoformans*, continued surveillance of resistance needs to be undertaken to limit the emergence of less susceptible strains of this yeast pathogen.

3. SEXUAL REPRODUCTION OF *CRYPTOCOCCUS NEOFORMANS*

In 1975 and 1976 the perfect state of the pathogenic yeasts *C. neoformans* and *C. gattii*, namely *Filobasidiella neoformans* and *Filobasidiella bacillispora*, were identified by Kwon-Chung. The culturing of clinical strains in differing combinations on various sporulation agar led Kwon-Chung to observe the development of hyphae with fused clamp connection, characteristic of basidiomycetes. Further observation revealed the development of hyphae into basidia and the production of basidiospores arranged in chain-like structures (Kwon-Chung, 1980).

3.1. Dikaryotic Mating in C. neoformans

During the sexual reproduction of heterothallic yeasts, generally one partner initiates a signal that results in the development of conjugation tubes of both partners. During the mating of *C. neoformans* however, the mating pheromone produce by MAT*a* (*MFa*) is expressed in response to nitrogen starvation; this induces the development of a conjugation tube in MATα cells. Cells of opposite mating types then fuse and form a heterokaryon that develops into dikaryotic hyphae possessing un-fused nuclei and fused clamp connections (Fig 2) (Kwon-Chung, 1980). Only once the hyphae have developed into basidia, will karyogamy and meiosis occur. The subsequent process of sporogenesis can be erratic; nevertheless it results in basidiospores of each mating type forming chain-like structures on the basidial head. Dispersion of each mating type is done in a random fashion and the basidiospores can be easily dislodged.

Interestingly, research has shown that the migration of mitochondrial DNA (mtDNA) is uni-parental, originating from the MAT*a* cell (Xu *et al*., 2000), a phenomenon observed in filamentous fungi but not among yeasts. The migration of the nucleolar genetic material appears to be unidirectional, moving along the conjugation tube of the MATα cell to the MAT*a* cell (Kwon-Chung *et al*., personal communication).

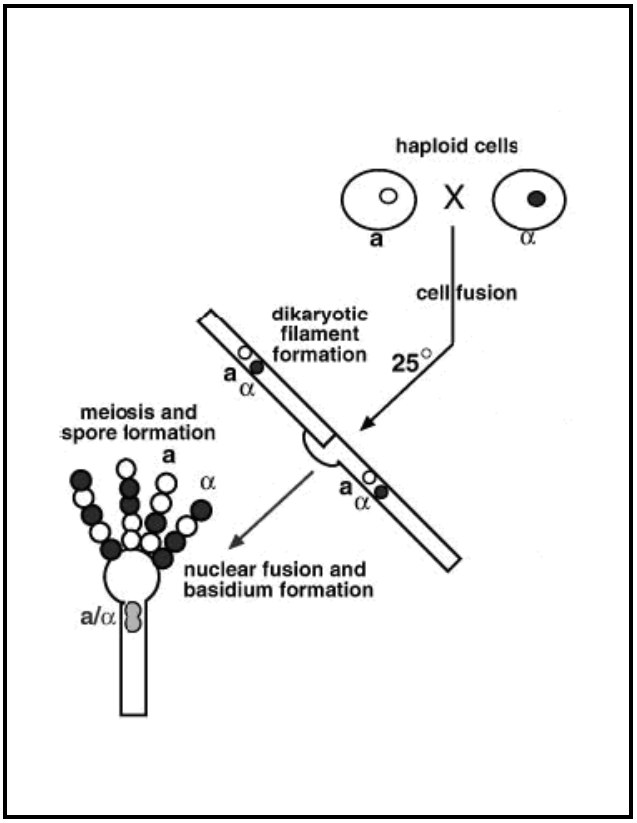

Figure 2 Dikaryotic mating of *C. neoformans* occurs when cells of opposite mating types fuse and form hyphae, with fused clamp connections, that ultimately give rise to basidia and basidiospores, the suspected infectious propagules (Hull and Heitman, 2002).

3.2. Monokaryotic Fruiting in C. neoformans

When haploid cells form true hyphae that give rise to basidia and basidiospores in the absence of the opposite mating type, the fruiting is termed monokaryotic fruiting (Esser and Meinhardt, 1977). Occurring in many higher basidiomycetes, such as the mushrooms, *C. neoformans* is the only known lower basidiomycete to undergo monokaryotic fruiting. Although hyphae can appear similar, monokaryotic hyphae can be distinguished firstly by the appearance of un-fused clamp connections, secondly the single haploid nuclei present within the hyphae and finally the development of long bead-like structures termed blastospores (Fig 3).

Despite rare reports of hyphal development by *C. neoformans* during infection (Bemis *et al*., 2000; Williamson *et al*., 1996; Neilson *et al*., 1978; Freed *et al*., 1971; Shadomy and Lurie, 1971; Shadomy and Utz, 1966) this pathogen is not regarded as being dimorphic as hyphal production was only observed under mating conditions or as a result of self-fertile diploid stains. In 1996, Wickes and co-workers were able to induce monokaryotic fruiting of MATα strains of *C. neoformans*. The phenomenon occurred under nitrogen starvation conditions at room temperature and resulted in the formation of basidia as well large numbers of viable basidiospores.

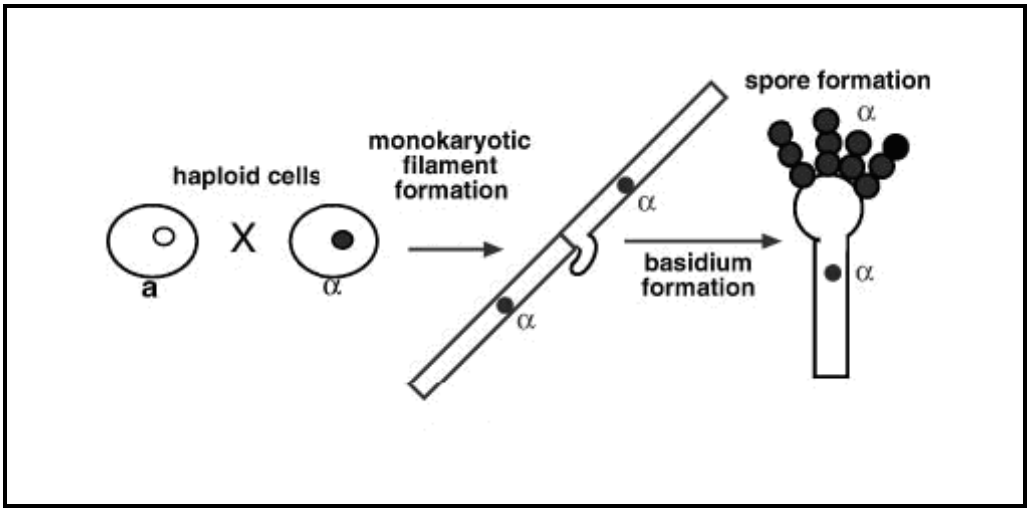

Figure 3 Monokaryotic fruiting of *C. neoformans* generally occurs when MATα cells form hyphae, with un-fused clamp connections, that ultimately give rise to basidia and basidiospores (Hull and Heitman, 2002).

The inability of MAT*a* strains to produce monokaryotic hyphae led the authors to conclude that the mating type bias of MATα cells observed in both environmental and clinical isolations may be as a result of monokaryotic fruiting. However, in 2003, Tscharke and co-workers identified two new strains of *C. neoformans* var. *neoformans* MAT*a* that were able to undergo monokaryotic fruiting. They also noted that *C. neoformans* var. *neoformans* (serotype D) strains were the most vigorous monokaryotic fruiters while *C. neoformans* var. *grubii* (serotype A) strains were poor monokaryotic fruiters or did not fruit at all. These findings would contradict the hypothesis that monokaryotic fruiting is responsible for the mating type bias as the majority of strains isolated are *C. neoformans* var. *grubii*, serotype A, MATα.

Interestingly, same sex mating of *C. gattii* MATα has been observed amongst strains isolated from the Vancouver Island outbreak (Fraser *et al*., 2005). Genotypic analysis of these

C. gattii strains revealed evidence of recombination; however, the offspring appeared to have descended from two MATα parents. Although the same phenomena has not been reported for *C. neoformans*, it may provide yet another alternative theory to the mating type bias observed in populations of both these pathogenic yeast species.

3.3. Genetic composition of the MAT locus of C. neoformans

In contrast to a number of basidiomycetes, *C. neoformans* has a bipolar mating system with two opposite mating types, MAT*a* or MATα (McClelland *et al*., 2002; Kwon-Chung, 1976). This first mating linked gene to be characterized from *C. neoformans* MATα was the mating pheromone (*MF*α) gene containing part of the MAT locus (Moore and Edman, 1993). In 1997 Wickes and co-workers were able to identify *STE12*α that showed homology to the *Saccharomyces cerevisiae STE12* gene; while in 2001 Chang and co-workers identified the corresponding *STE12a*. Subsequent mapping of the MATα locus revealed that presence of several MATα-specific homologs of pheromone response mitogen activated protein (MAP) kinase cascade genes, a myosin gene as well as the translation initiation factor *PRT*α (Karos *et al*., 2000). The presence of the latter two genes is unusual as genes unrelated to mating type are not located in the MAT locus of other fungi.

Sequence data of the MAT loci from both mating types has shown that both loci contain more than 20 genes however; the MAT*a* locus is approximately 120 kb while the MATα is only 100 kb (Lengeler *et al*., 2002). To date no other heterothallic fungus has revealed the same genetic organization of the MAT locus as seen in *C. neoformans*.

3.4. Pheromone Response Pathway of C. neoformans

Opposite cell types of *C. neoformans* are able to signal and respond during mating through a conserved-pheromone receptor system (Wickes, 2002). The pheromones of each mating type, designated *MFa* for MAT*a* pheromone and *MF*α for MATα pheromone, are small multi-copy hydrophobic peptides (McClelland *et al*., 2002). Three genes have been identified that encode for the *MF*α pheromone, termed *MF*α*1*, *2* and *3* (Shen *et al*., 2002; Davidson *et al*., 2000; Moore and Edman, 1993), and all are induced under nutrient limiting conditions and co-culture with *MATa* cells. Similarly, 3 related genes, sharing little amino acid sequence homology with *MF*α (McClelland *et al*., 2002), encoding for the *MFa* pheromone have also been identified. However unlike *S. cerevisiae*, the deletion of the pheromone genes does not inhibit the mating or spore production of *C. neoformans*.

Mating is initiated when a pheromone binds to a cognate receptor subsequently leading to the activation of a MAP kinase cascade of which some components appear to be mating type specific (Lengeler *et al*., 2000; Wang and Heitman, 1999). In MATα cells, the *MFa* pheromone activates the MAP kinase cascade by binding to a G-protein coupled receptor and a heterotrimeric G-protein (Fig 4). The Gβ-subunit tranduces the signal to Ste20α, a PAK-kinase that is known to be mating specific (Wang *et al*., 2002; Wang *et al*., 2000). The PAK kinase relays the signal to the MAP kinase cassette comprised of Ste11α, Ste7 and Cpk1 (Clarke *et al*., 2001). It is speculated that these signals induce the transcription factor Ste12α, to control fruiting and virulence as well as an identified mating control factor. Once again, unlike *S. cerevisiae*, the deletion of the transcriptional activator genes (*STE12a* and *STE12*α), does not affect the fertility of *C. neoformans*, however efficiency of monokaryotic fruiting, capsule and melanin production are reduced, indicating that *STE12* may be necessary for other virulence factors (Chang *et al*., 2000; Yue *et al*., 1999).

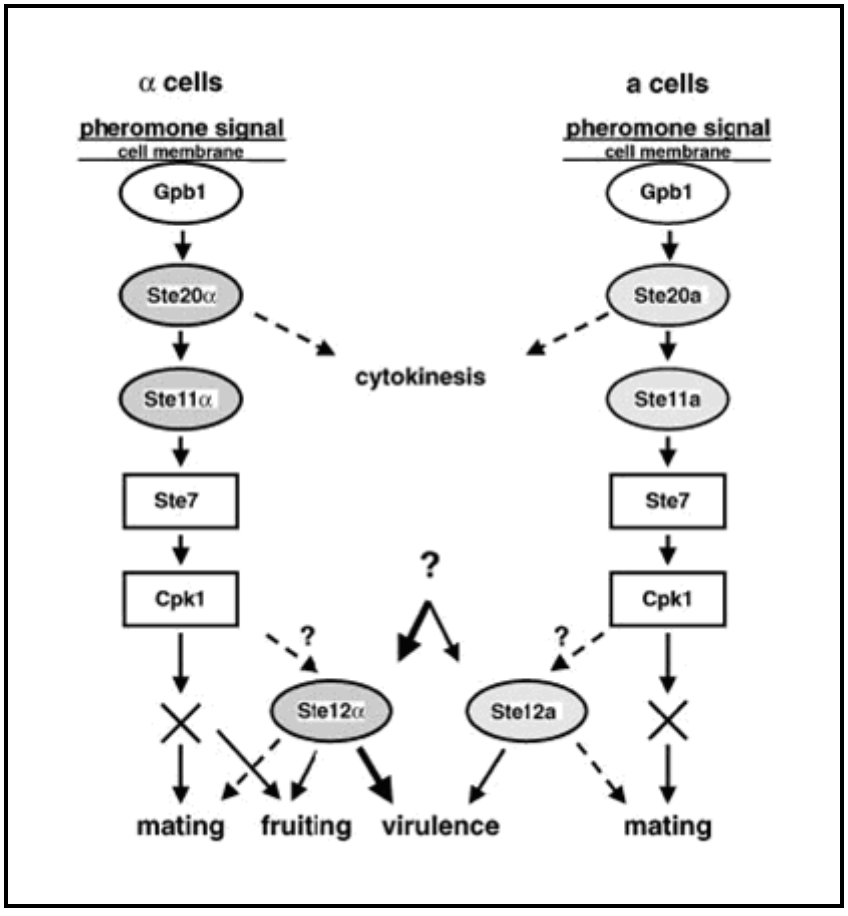

Figure 4 The pheromone signalling pathway of *C. neoformans* is conducted via a MAP kinase cascade (Hull and Heitman, 2002).

3.5. The Link between Mating Type and Virulence

The speculation that mating type may be linked to virulence was the result of a high isolation frequency of MATα strains from cryptococcosis cases with reported ratios as high as 30:1 (Halliday *et al*., 1999). In 1992, Kwon-Chung and co-workers constructed a pair of congenic MAT*a* and MATα strains for *C. neoformans* var. *neoformans* (serotype D), B-4476 (MAT*a*) and B-4500 (MATα). The virulence of these congenic strains was tested using the murine tail vein model. Mice infected with B-4500 displayed a higher mortality rate that generally occurred within in shorter time period, thus indicating that MATα is indeed more virulent. It should be noted however that the killing of mice by MAT*a* cells did occur, albeit at lower levels, indicating that although this mating type is less virulent, it is still lethal.

Interestingly, virulence testing conducted using congenic MAT*a* and MATα strains, KN99-*a* and KN99-α, for *C. neoformans* var. *grubii* (serotype A) showed equivalent virulence between the two mating types in two separate animal models (Nielsen *et al*., 2003). Such virulence testing may have revealed genetic diversity between the differing serotypes of *C. neoformans* rather than mating type.

Today an increase in the number of environmental isolations of *C. neoformans* has revealed the same mating type bias exists within naturally occurring populations of this pathogen. Indeed ratios as high as 40:1 have been reported for environmental isolations (Halliday *et al*., 1999), but rare cases of even distribution between mating types have been reported. In 2006, Litvintseva and co-workers reported an unusually high proportion of fertile *C. neoformans* MAT*a* isolates in Botswana. A closely related species to *C. neoformans*, *C. gattii*, demonstrates the same dominance with regards to uneven mating type distribution, although cases of even ratios have also been reported. In 1999 Halliday and co-workers reported the isolation of *C. gattii* with ratios of approximately 1:1 with regards to mating type (Halliday *et al*., 1999).

Researchers therefore argue that a higher frequency of exposure to naturally occurring populations of MATα strains of *C. neoformans* could explain the mating type bias observed within clinical isolations. However, environmental numbers raises the question of whether or not MATα is better suited for survival than its counterpart. Although no clear explanations are given, researchers have suggested that sex-linked lethal mutations associated with MAT*a* could explain the dominance of MATα. Alternatively, the occurrence of clonal populations of *C. neoformans*, as well as the closely related species *C. gattii* known to display the same mating type bias, may indicate that mating is not essential for survival (Franzot *et al*., 1997; Brandt *et al*., 1996; Chen *et al*., 1995).

Interestingly, a number of fungal pathogens have retained genes essential for sexual reproduction, such as *Candida albicans* and *Apergillus fumigatus*, however like *C. neoformans*; their populations are predominantly clonal showing limited recombination (Heitman, 2006). This limited sexual reproduction appears to act as a virulence strategy enabling fungal pathogens to survive within both a host and an environmental niche. However, alternative theories to explain the mating type bias seen within *C. neoformans* populations need to be explored further**.**

4. VIRULENCE FACTORS OF *CRYPTOCOCCUS NEOFORMANS*

Virulence can be defined as a microbial characteristic that is expressed only in susceptible hosts and involves the microbe's capacity to cause damage to the host itself (Casadevall *et al*., 2003; Steenbergen *et al*., 2003). There are three basic criteria that must be considered in order to define a specific microbial characteristic as a virulence factor. Firstly, the characteristic must be associated with the pathogen. Secondly, the inactivation of the associated gene must decrease the overall virulence. Finally, the complementation or restoration of the gene product must restore virulence (Steenbergen *et al*., 2003). Although the precise mechanisms of virulence remain unresolved, what is clear is the ability of *C. neoformans* to avoid the humoral response. This phenomenon is not the result of one virulence factor, but rather the cumulative effect of a number of virulence factors.

For this reason, there are a number of virulence factors associated with the pathogenesis of *C. neoformans*, but perhaps the best understood include laccase and melanin synthesis, the ability to withstand physiological temperatures as well the production of a polysaccharide capsule (Steen *et al*., 2002; Zhu *et al*., 2001; Liu *et al*., 1999). Other virulence factors that need to be considered include phospholipase, urease and proteinase production and as previously discussed the mating type of the yeast itself.

4.1. The Polysaccharide Capsule

While serving as a major diagnostic feature (Bose *et al*., 2003) the polysaccharide capsule of *C. neoformans* plays a vital role in the virulence of this pathogen by inhibiting effective phagocytosis and clearance of the yeast by macrophages. As a result, *C. neoformans* is able to persist and multiply within human macrophages however; infections appear to only become life-threatening when the human immune system is compromised (Goldman *et al*., 2001).

4.1.1. Polysaccharide Capsule Structure

The capsule is regarded as the main virulence factor of *C. neoformans* (Janbon 2004). At least four genes have been identified (CAP10, CAP59, CAP60 and CAP64) and all are required for virulence (Janbon 2004; Steenbergen *et al*., 2003). The capsule is composed of a minimum of three components namely, mannoprotein; galactoxylomannan (GalXM) and glucuronoxylomannan (GXM) (Janbon, 2004; Bose *et al*., 2003; Steenbergen *et al*., 2003). The latter component (GXM) composes approximately 90% of the capsule structure and consists of mannose residues that are α-1,3 linked with xylosyl and glucuronyl side groups (Janbon, 2004; Bose *et al*., 2003). Certain mannose residues are 6-O-acetylated usually with an un-branched mannose however, substitution with glucuronic acid does occur. It is the variation of xylose addition and acetylation of GXM, along with antibody binding that allows the classification into the four different serotypes, namely serotypes A and D for *C. neoformans* var. *grubii* and var. *neoformans* respectively and serotypes B and C for *C. gattii*. This classification however does not take into account the variation within GXM structure that appears to correlate directly with the range of virulence. It has therefore been suggested that the yeast be classified on the basis of minimum GXM repeating unit in order to determine the virulence of the isolated strain.

The second structural component, GalXM, comprises only 7 % of the polysaccharide capsule (Bose *et al*., 2003; Janbon, 2004). It consists of a α-1,6-linked galactose polymer with a number of side chains of varying lengths. These side chains can consist of a number of structures including galactosyl, mannosyl and xylosyl residues. Once again structures vary between the four different serotypes.

The final component, mannoprotein, is perhaps the most vital with regards to the host's immune response. These proteins are responsible for the induction of cell-mediated immunity and cytokine production, both of which are critical during the initial stages of infection (Janbon, 2004; Bose *et al*., 2003).

4.1.2. Regulation of Capsule Biosynthesis

Capsule regulation appears to be dependent on a number of varying factors (Janbon, 2004). Nutrient availability appears to play a major role as capsule size is dependent on the available carbon source, amino acids and vitamins. High glucose concentrations inhibit capsule synthesis while low concentrations of glucose, mannose, xylose and sucrose in combination with the amino acids thiamine, L-proline and asparagine enhance capsule production (Janbon, 2004). During infection capsule size is also dependent upon the organ

infected (Rivera *et al*., 1998). Isolates originating from lung tissue were shown to have on average thicker capsules than those originating from brain tissue. Rivera and co-workers suggest that this difference in capsule size between isolates originating from both lung and brain tissue is as a result of the higher iron concentration found within brain tissue. They argue that the higher concentrations of this metal may inhibit capsule production during infection however the exact mechanisms of inhibition is unknown.

To date signal transduction pathways involved in capsule synthesis are not yet fully understood however, a number have been implicated in the process of capsule regulation (Janbon, 2004). Firstly, transcription and translation of capsule genes appears to be regulated by the target of rapamycin (TOR) pathway that responds to changes in availability of nitrogen and amino acids. Secondly, response to changes in osmotic pressure could potentially be controlled by the high osmolarity glycerol (HOG) pathway as is seen in *S. cerevisiae*. And finally the cyclic adenosine monophosphate (cAMP) pathway is involved in the regulation of capsule biosynthesis although the surface receptors and a number of lower protein kinase targets are yet to be identified.

4.1.3. Role of the Polysaccharide Capsule during Pathogenesis of *C. neoformans*

Traditionally the capsule was thought to be primarily anti-phagocytic, but a number of additional factors have been resolved and include the alteration of antigen presentation, inhibition of cytokine production, reduction of leukocyte migration to inflamed sites, depletion of complement components and macrophage dysfunction (Janbon, 2004; Bose *et al*., 2003; Steenbergen *et al*., 2003; Buchannan *et al*., 1998). Once engulfed, the yeast exudes polysaccharides from its capsule into vesicles around the phagosome that gradually accumulate in the cytosome resulting in dysfunction of the macrophage and possibly death (Steenbergen *et al*., 2003). More recently however, is the implication that the capsule enables the yeast to replicate within the macrophages allowing infections to remain dormant and be "re-activated" at a later stage (Janbon, 2004; Goldman *et al*., 2000). In fact ingested yeast cells appear to replicate as rapidly as extra-cellular cells with a single macrophage containing an average of 30 to 40 yeast cells (Tucker and Casadevall, 2002). Although the exact mechanism of yeast replication within macrophage cells remains unclear, Tucker and Casadevall have suggested that the larger capsule present in lung tissue potentially dilutes lysosomal contents and serves as both a physical barrier and creates separation between the surface of the yeast cell and the microbiocidal compounds released from the phagosomal membrane (Tucker and Casadevall, 2002). This increase in fungal burden allows the yeast to

disseminate via macrophages and blood monocytes throughout the human body resulting in the various manifestation of cryptococcosis.

4.2. Laccase

Laccases are a large group of enzymes termed the multi-copper or blue oxidase enzymes and include ascorbic acid oxidase (Mayer *et al*., 2002; Liu *et al*., 1999; Thurston; 1994). The enzyme is regarded as being ubiquitous in nature and has so far been identified in all domains of life, although the majority have fungal origins (Claus, 2004). Laccases are also non-specific regarding their substrate; however enzyme activity varies from laccase to laccase (Thurston, 1994). Generally any substrate that is similar to *p*-diphenol will be utilized by this group of enzymes (Mayer *et al*., 2002).

4.2.1. The Cryptococcal Laccase Enzyme

The laccase produced by *C. neoformans* was originally thought to be a phenol or diphenol oxidase due to its ability to produce coloured pigments (Liu *et al*., 1999; Thurston; 1994). Atomic absorption revealed that the enzyme contained 4 mol/mol of copper and had an absorbance of 610 and 320 nm, both of which are characteristic of type I and III copper laccases respectively (Zhu and Williamson, 2004). This evidence, combined with the presence of several copper binding sites within the amino acid sequence derived from the laccase (*CNLAC1)* gene, indicating that the enzyme was indeed a fungal laccase. Recent genome projects revealed that *C. neoformans* possess a second laccase gene, *CNLAC2*. Present in the form of a tandem repeat and in the same orientation as *CNLAC1*, the two genes share 65 % nucleotide homology; but deletion of the *CNLAC1* gene abolishes enzyme activity indicating that the originally discovered gene is in fact dominant over its counterpart (Zhu and Williamson, 2004).

Interestingly, evolutionary studies conducted by Valderrama and co-workers (2003), suggests that the laccase of *C. neoformans* is in fact not a true fungal laccase. While the majority of laccases are extremely diverse in terms of their protein structure and substrate utilization, their catalytic sites are regarded as being relatively conserved (Mayer *et al*., 2002). Valderrama and co-workers (2003) conducted phylogenetic studies by comparing the active sites of various laccase enzymes and noted that the laccase expressed by *C. neoformans* and *Aspergillus nidulans* fell within their own clade away from the other groups. It is therefore suggested that cryptococcal laccase is not a true laccase but rather a representative of a different family of multi-copper oxidases (Valderrama *et al*., 2003).

4.2.2. Molecular Regulation of the Cryptococcal Laccase Enzyme

Molecular regulation of the cryptococcal laccase is thought to have evolved due to environmental as opposed to physiological pressures (Zhu *et al*., 2004). For example, glucose limited conditions, such as those present in the brain, stimulates the expression of laccase (Salas *et al*., 1996). Metal induction of the cryptococcal laccase has been well characterized with regards to copper where quantities as low as 5 µM resulted in an increase in laccase transcription (Zhu *et al*., 2004).

However, the regulation of *CNLAC1* in *C. neoformans* contains features usually associated with regulation in higher eukaryotes (Zhang *et al*., 1999). The transcriptional regulation in higher eukaryotes, both mammalian and plants, is usually characterised by the presence of multiple interacting DNA binding sites found over a large upstream region of genes, as well as the ability to use enhancers, such as Sp1, that are rich in glutamine. In contrast, fungal transcriptional regulation contains fewer transcriptional elements that are generally located closer to the open reading frame (ORF).

Zhang and co-workers evaluated the 5´-upstream region *CNLAC1* under glucose repression in order to identify any enhancement and repression regions. Studies revealed two upstream enhancer regions, one of which contains a consensus Sp1 DNA binding site (Zhang *et al*., 1999). Further analysis revealed that Sp1 DNA binding sites are also present in other genes, namely CAP 64 and CAP 59 that are involved in polysaccharide capsule synthesis. This suggests a co-regulation of these virulence factors by a transacting Sp1 protein.

The second enhancer region contained an E2F consensus site, a gene family that is predominantly associated with regulation of cell growth (Zhang *et al*., 1999). It is suggested that the E2F gene is needed to synchronize genes during the cell cycle in order to prevent the uptake of iron during the log phase. The uptake of iron at this stage would prove lethal to cell due to the ferrioxidase activity of the laccase enzyme.

The large number of repressor and enhancer sites implies that the enzyme is under strict regulation in order to respond to both environmental and host stimuli varying expression under altering conditions (Zhu and Williamson, 2004).

4.2.3. Cellular Location of the Cryptococcal Laccase Enzyme

Studies have shown that the laccase enzyme appears to be localized towards the outer region of the cryptoccocal cell wall (Zhu *et al*., 2001). From this position, the enzyme is able to interact directly with the host's immune system and other extracellular products. A second advantage of such a location is that substrate transporters, such as those required for

dopamine and other catecholamines, are made redundant. It must also be noted that the extracellular production of oxidation products may explain why the production of melanin *in vitro* requires levels of dopamine that are much higher than levels present within brain tissue.

4.2.4. Role of the Laccase Enzyme during Pathogenesis of *C. neoformans*

Originally thought to only produce the virulence factor melanin, recent studies indicated that the laccase enzyme itself is in fact a potent virulence factor (Zhu *et al*., 2001; Liu *et al*., 1999). Liu and co-workers compared macrophage-mediated killing between both laccase-positive and laccase-deficient strains. Their study was able to show that the laccase enzyme confers protection without the presence of melanin but rather by an alternative enzyme activity (Liu *et al*., 1999).

As mentioned previously, fungal laccases belong to the family of copper or blue oxidase enzymes. Recently the iron transporter Fet3, isolated from *S. cerevisiae*, has been shown to be a member of this family (de Silva *et al*., 1997). The oxidation of iron from Fe(II) to Fe(III) is coupled to the transport of iron across the plasma membrane by the Fet3 protein. The cryptococcal laccase shares this iron oxidase activity with Fet3 (Liu *et al*., 1999). Interestingly, macrophages require Fe(II) to produce toxic oxygen metabolites that are essential for macrophage mediated killing of microbes (Zhu *et al*., 2001). These oxidative bursts would be less efficient against *C. neoformans* as the cryptococcal laccase enzyme would compete for the substrate Fe(II) due to its iron oxidase activity, ultimately aiding the pathogen to avoid the human immune system.

4.3. Melanin

Melanin is a negatively charged pigment that is ubiquitous in nature and is produced by a number of organisms that includes bacteria, fungi, plants and animals (Hill, 1992). Although their structures are poorly understood, melanins are described as macromolecules that are insoluble, resistant to acid degradation and form a stable free radical population. Recently however, melanin have been found to play an essential role is the pathogenesis of many fungal pathogens such as *Aspergillus fumigatus*, *C. neoformans*, *C. gattii*, *Mycobacterium leprae*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii* and *Wangiella dermatitidis* with the two most important being dihydroxynaphthalene (DHN) melanin and dihydroxyphenylalanine (DOPA) melanin (Langfelder *et al*., 2003).

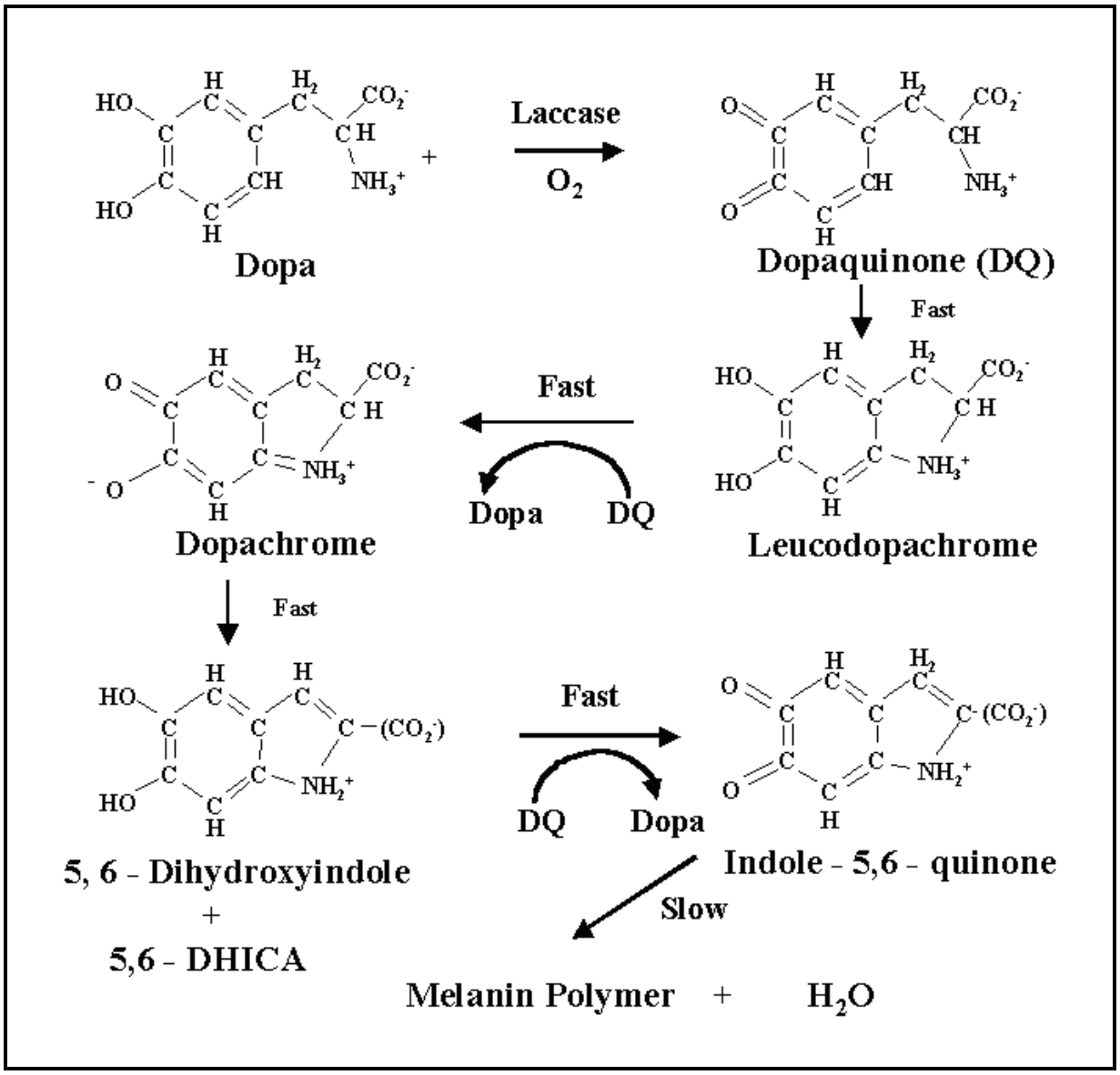

Figure 5 Proposed melanin synthesis scheme in *Cryptococcus neoformans* adapted from the Mason-Raper model (Williamson, 1997).

4.3.1. Biosynthesis of Melanin in *C. neoformans*

In 1986, Bell and Wheeler proposed the biosynthesis pathway of DOPA-melanin that strongly resembled the pathways observed in mammals. With the well known neurotropism of *C. neoformans*, the Mason-Raper scheme for the biosynthesis of eumamalian melanin was adapted using DOPA (Williamson, 1997) (Fig 5). Following this pathway, DOPA is oxidized by the laccase enzyme to form the intermediate dopachrome. Dopachrome then decarboxylates non-enzymatically to form 5,6-dihydroxyindole (DHI) and 5,6 dihydroxyindole carboxylic acid (DHCI). Further oxidation to form indole-5,6-quinone is followed by polymerization to melachrome and finally melanin that becomes covalently liked

to the cell wall. It should however be noted that the Mason-Raper model of melanin biosynthesis is based on the mammalian enzyme tyrosinase, capable of only oxidizing tyrosine while laccase enzymes display a much broader substrate specificity.

4.3.3. The Role of Melanin during Pathogenesis of *C. neoformans*

In 1982, Kwon-Chung and co-workers were able to demonstrate that the infection of mice with wild-type melanin positive *C. neoformans* strains (Mel^+) proved to be fatal. Mice infected with strains unable to produce melanin (Mel^-) however, survived and showed clearance of *C. neoformans* cells from the spleen, liver and brain. Similar results were observed by Rhodes and co-workers with regards to mortality induced by Mel^+ and Mel^- *C. neoformans* strains. Interestingly, in mice that died after infection with Mel^- strains, approximately 50 % of cells isolated had reverted to the wild-type phenotype. With the isolation and characterization of *CNLAC1* and the subsequent construction of congenic strains (Salas *et al*., 1996), the significant reduction of virulence seen in the Mel^- strains during the intravenous mouse model indicates melanin plays an important role in the virulence of *C. neoformans*.

Despite this fact, the mechanism of how melanin enhances the virulence of *C. neoformans* remains unclear. Cryptococcal melanin has been implicated in the maintenance of cell wall integrity and the protection of the yeast cell against a number of factors that include ultraviolet light (Wang *et al*., 1994), temperature fluctuations (Rosas *et al*., 1997) heavy metals (Garcia-Rivera *et al*., 2001), oxidants (Williamson, 1997), enzyme degradation (Rosas *et al*., 2001), microbial peptides and anti-fungals (Doering *et al*., 1999), anti-fungal therapy (Ikeda *et al*., 2003) as well as phagocytosis (Steenbergen *et al*., 2001).

4.4 Thermo-tolerance

In order for a pathogen to cause disease it must be able to withstand and proliferate at physiological temperatures (Kraus *et al*., 2004). Indeed growth at 37°C often results in a phenotypic switch in many pathogenic yeast species resulting in an increase in virulence. It is however interesting to note that approximately 270 fungal species are known to cause disease within humans and the majority, the dermatophytes, primarily affect the skin and nails. These regions are considered to have a lower temperature than the remaining body high-lighting the pathogen's struggle in surviving at higher temperatures (Perfect, 2006).

Interestingly, *C. neoformans* var. *neoformans*, *C. neoformans* var. *grubii* show great diversity with regards to their thermo-tolerance. Currently, serotype A is regarded as

being more thermo-tolerant, and is isolated from 90 % of all clinical cases world-wide (Martinez *et al*., 2001; Mitchell and Perfect, 1995). Perhaps the only exception to this trend exists within northern European countries where *C. neoformans* var. *neoformans* (serotype D) displays a greater prevalence (Martinez *et al*., 2001; Dromer *et al*., 1996).

This observation has been attributed to the more temperate climate of these northern European countries. Martinez and co-workers (2001) were able to demonstrate that serotype D was on average more susceptible to heat killing than serotype A. On average, at temperatures of 41 ºC and higher, *C. neoformans* var. *grubii* (serotype A) displays greater thermal tolerance than *C. neoformans* var. *neoformans* (serotype D). A second case study, involving the simultaneous infection by *C. neoformans* and malaria, noted that during relapses of malaria, *C. neoformans* cells were cleared from the spinal fluid (Kligman *et al*., 1949). Researchers have speculated that the fever induced by the malaria may have resulted in the elimination of *C. neoformans* cells. This thermo-intolerance displayed by *C. neoformans* var. *neoformans* could account for its higher prevalence in European countries, with temperate climates, as well as the high predilection seen for skin tissue that is regarded as having a cooler overall temperature (Martinez *et al*., 2001; Dromer *et al*., 1996).

4.5. Additional virulence factors

A variety of additional virulence factors have been observed during the pathogenesis of *C. neoformans* and include phospholipase, urease and proteinase expression.

4.5.1. Phospholipase

Phospholipases are capable of hydrolyzing ester bonds that aid during cell lysis and disruption of the cell membrane. *C. neoformans* secretes a phospholipase, that is controlled by a single gene termed phospholipase B1 (*PLB1*) and is suspected of aiding in the spread of the pathogen as *plb1*$^{-}$ *C. neoformans* strains were unable to degrade the phagosome membrane thereby limiting the dispersion of the yeast (Cox *et al*., 2001).

Interestingly, comparison of phospholipase activity amongst environmental and clinical isolates of *C. neoformans* revealed that the clinical strains produced significantly more of the enzyme (Ghannoum, 2000). This would indicate that phospholipase expression serves a vital role during the pathogenesis of *C. neoformans*.

4.5.2. Urease

Urease is able to convert urea to ammonia and carbonate resulting in a localized increase in pH (Casadevall and Perfect, 1998). To date the majority of *C. neoformans* strains have tested positive for urease expression while urease mutant strains of *C. neoformans* have displayed decreased pathogenicity (Cox *et al*., 2000).

4.5.3 Proteinase

Proteinases are regarded as important virulence factors in both fungi and bacteria (Ruma-Hayens *et al*., 2000; Brueske *et al*., 1986). Cryptococcal proteinases are suspected to provide nutrients for the pathogen by degrading proteins such as elastin, collagen, fibrinogen and immunoglobin although a clear genetic link to virulence has not yet been established (Chen *et al*., 1996).

5. ORIGINS OF VIRLENCE IN *CRYPTOCOCCUS NEOFORMANS*

The development and subsequent maintenance of virulence factors in *C. neoformans* is largely attributed to natural selective pressures. Consequently, these virulence factors are commonly termed “dual-use” virulence factors as it has been proposed that factors such as capsule production and laccase expression evolved to ensure the survival of *C. neoformans* within its ecological niche and not survival within a human host.

5.1. Microbial Interactions

Despite the inability to pin-point the exact ecological niche of *C. neoformans*, it is certain the pathogen participates in a number of different interactions, both positive and negative, within the microbial community (Allee *et al*., 1949). Negative reactions limit overall population densities by serving as feedback mechanisms and ultimately prevent over population and destruction of a favourable niche. Positive interactions combine physical and metabolic capabilities within a microbial community that both enhance population growth and improve the organism’s ability to thrive in a previously unfavourable niche. Positive interactions would be regarded as commensalisms, synergism and amensalism while negative interactions include competition and amensalism. Parasitism and predation are considered to be both positive and negative towards a given population, of which the latter plays a vital role in the acquisition and maintenance of the virulence factors of *C. neoformans*.

5.2. Predation

There is a fine line between what is regarded as parasitism and what is regarded as predation (Atlas and Bartha, 1998). Some would argue that the bacterium *Bdellovibrio* is in fact an ecto-parasite as it does not engulf the susceptible bacteria (Riitenberg, 1983). Others suggest that since *Bdellovibrio* shows no host specificity, except towards gram-negative bacteria, it is in fact a predator (Slobodkin, 1968). However, co-existence of predator and prey relies on the latter's ability to avoid and survive predation. Many microorganisms produce cell structures, such as spores, that are simply resistant to lyses and digestion by the predator (Kuhlman and Heckman, 1985). Others are able to modify their overall cell structure making engulfment difficult. Environmental structures, such as clay particles, also play a role by physically preventing predation.

Early studies indicated that the amoeba, *Acanthamoebae polyphaga,* was capable of engulfing large numbers of *C. neoformans* cells that ultimately resulted in the killing of the yeast cell (Neilson *et al*., 1978). Further research conducted by Steenbergnen (2001) and Mylonakis (2002) revealed that the amoeba *A. castenalli* and the nematode *Caenorhabditis elegans* were also capable of ingesting the pathogen *C. neoformans*. However, unlike *A. polyphaga*, ingestion resulted in the death of the amoebae and nematodes and not the yeast *C. neoformans*. Indeed *C. elegans* was shown to be capable of ingesting other yeast namely *C. laurentii*, *C. kuetzingii*, however death only occurred after ingestion of *C. neoformans.*

Interestingly, the interactions of these predators and *C. neoformans* closely resembled the interactions observed between macrophages and this yeast pathogen. As observed with macrophages, acapsular yeast cells are more readily ingested by the predators, but engulfment of encapsulated *C. neoformans* cells does occur. Once ingested, the yeast cells are internalized and enclosed within a membrane bound vacuole. Here *C. neoformans* is able to replicate and the formation and accumulation of polysaccharide vesicles occurs. Contact with these vesicles results in cell membrane leaking and ultimately lysis of the amoeboid cell.

Unfortunately, the current research does not indicate whether *C. neoformans* shows an increase in virulence after passage through both amoebae and nematodes. Changes in virulence is often associated with phenotypic switching, a characteristic displayed by *C. neoformans* (Guerrero *et al*., 2006). Such phenotypic switching is a result of micro-evolution and is thought to occur during the course of infection aiding the pathogen to avoid an already compromised immune system. Alternatively, micro-evolution may be as a result of predation by amoebae and nematodes on *C. neoformans* ultimately inducing limited genetic diversity within a predominantly clonal population (Halliday *et al*., 2003; Trilles *et al*., 2003).

6. FINDING THE TRUE ECOLOGICAL NICHE OF *CRYPTOCOCCUS NEOFORMANS*

As mentioned earlier, the re-classification of *C. gattii* as a new species was supported by the fact that *C. neoformans* and *C. gattii* were isolated from different habitats. *C. gattii* occurs predominately in sub-tropical and tropical regions (Sorrell *et al*., 1997) and has been shown to have a strong ecological association with a number of *Eucalyptus* trees, particularly *Eucalyptus camaldulensis*, generally known as the river red gum, and *Eucalyptus tereticornis*, commonly known as the forest red gum. Both these species are prevalent in Australia where a high incidence of cryptococcosis is observed amongst native animal and human populations (Halliday *et al*., 1999). By means of random amplified polymorphic DNA (RAPD) and PCR fingerprinting, Sorrell and co-workers (1996) were able to determine that the source of these infections was indeed the *Eucalyptus* species as the genetic composition of those pathogens found on the trees matched those pathogens isolated from infected animal and human populations.

Despite this perceived association, the role that the tree plays in the life cycle of the yeast remains unclear. Halliday and co-workers (2003), suggested that the association between *C. gattii* and the *Eucalyptus* species is not as relevant as previously thought. They argue that the lack of genetic diversity amongst *C. gattii* species found on *Eucalyptus* species in Australia indicates wide-spread clonality and therefore *C. gattii* is capable of utilizing the favourable environment offered by the decaying wood of these *Eucalyptus* species. Interestingly, different trees harboured genetically distinct populations of *C. gattii* indicating that there was no dispersal of the pathogen between trees.

Indeed as Halliday suggests, these ecological associations are not definite. There are a number of different tree species from which the yeast has been isolated. In Brazil, *C. gattii* has been isolated from the pink shower tree, the fig tree as well as the pottery tree, in Columbia the almond tree has also been colonized while on Vancouver Island, a temperate climate, native Douglas fir, maple and Garry oak trees appear to be the preferred habitat (Fraser *et al*., 2003; Halliday *et al*., 2003). Researchers have also managed to isolate *C. gattii* and *C. neoformans* from a number of different tree species within the Amazon forest, suggesting that the two species are rather associated with decaying wood as opposed to any particular tree species in this large area (Trilles *et al*., 2003; Fortes *et al*., 2001; Randhawa *et al*., 2000; Lazéra *et al*., 1996).

The variants *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* have a worldwide distribution and were originally thought to be associated primarily with avian guano or avian guano contaminated soil (Sorrell *et al*., 1997). The urease enzyme of *C. neoformans* is able to convert urea within the avian guano into ammonia, a form of nitrogen that can readily be assimilated (Cox *et al*., 2000). However, due to the high internal temperature of the birds, approximately 42 ºC, the birds themselves do not develop cryptococcosis.

Strains *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* have been isolated from a variety of environmental sources including soil, vegetative debris and particularly decaying wood and the hollows of trees (Trilles *et al*., 2003; Randhawa *et al*., 2001; Lazéra *et al*., 1996). The latter source appears to be promising with regards to locating the ecological niche of *C. neoformans* as the pathogen produces a laccase enzyme, known as a potent virulence factor as well a ligninolytic enzyme (Min *et al*., 2001). The laccase enzymes are polyphenol oxidases that are capable of oxidizing phenolic compounds and aromatic amines. Unlike the basidiomycetous white rot fungi that secrete laccase, the cryptococcal laccase is covalently bound to the outer membrane of the yeast cell (Zhu *et al*., 2001). This localization may help facilitate the survival of *C. neoformans* within a woody environment. Lignin is a large polymer, unable of traversing the cell membrane. The membrane localization of laccase would therefore allow direct contact between the yeast cell and the lignin thereby facilitating degradation of the polymer in close proximity to the cell. A similar phenomenon occurs among biofilm associated micro-organisms, where it is known that the localization of exo-enzymes, within exo-polymer capsules, enhances the competitiveness of these micro-organisms in oligotrophic environments (Decho, 1990).

The laccase enzyme of *C. neoformans*, coupled to the recent discovery of a cryptococcal cellulase (Loftus *et al*., 2005), further links *C. neoformans* to a potentially woody ecological niche. However, research conducted by Trilles and co-workers (2003) revealed that unlike *C. gattii*, *C. neoformans* isolates originating from neighbouring trees shared considerable genetic homogeneity, indicating very little genetic recombination as well as dispersal occurs.

7. CONCLUSIONS

Cryptococcus neoformans is a pathogenic basidiomycete (Boekhout *et al*., 1997), responsible for approximately 10 % of cryptococcosis infections associated with patients

already suffering with HIV and AIDS (Chuck and Sande, 1989) as well as 5 % of all organ transplant recipients (Vilchez *et al*., 2003; Husain *et al*., 2001). In many cases cryptococcosis is the first indication of AIDS and is the leading mycological cause of mortality among these patients (Rozenbaum and Rios Goncalves, 1994; Kovacs *et al*., 1985).

With a relatively simple nutrition, this yeast is able to reproduce both asexually and sexually, however, *C. neoformans* does not require any animal, plant or protists host. The identification of ligninolytic (Min *et al*., 2001) and cellulolytic (Lotus *et al*., 2005) enzymes coupled to the repeated isolation of *C. neoformans* from decaying wood and the hollows of trees (Trilles *et al*., 2003; Randhawa et al., 2000; Lazéra *et al*., 1996) gives a strong indication of a suspected woody niche for this pathogen. Although not conclusive, we can assume that the niche of *C. neoformans* is extraordinary complex and probably includes a host of other organisms including other fungi, bacteria, protists, animals and plants.

Studies have shown that within sub-Saharan Africa, up to 30 % of AIDS patients suffer from cryptococcosis (Powderly, 1993). A recent survey conducted within the Gauteng Province, South Africa, by the Gauteng Cryptococcal Surveillance Group during 2002 and 2003 were able to identify 1195 cryptococcosis cases during the first year (McCarthy *et al*., 2003). Cryptococcal meningitis accounted for 95 % of the cases, and the survey determined a mortality rate of 31 %. These relatively high mortality rates emphasize the need to further examine this ubiquitous yeast pathogen to determine is true ecological niche in order to limit exposure and perhaps prevent infection of an increasing vulnerable HIV and AIDS population.

8. PROJECT OBJECTIVES

Therefore with the above as background the objective of this study is three-fold:

1. By means of a preliminary study, we hope to obtain an indication of the prevailing *C. neoformans* genotypes occurring within the HIV positive and AIDS populations of South Africa and to obtain an indication of its distribution within the environment, particularly within the Western Cape.
2. To examine whether clinical and environmental isolates of *C. neoformans* var. *grubii* are capable of growth and interaction with selected microbes when cultured on woody debris.
3. And finally, to examine the hypothesis that *C. neoformans* is capable of producing all its ontogenic stages on woody debris.

Please note the following results chapters have been written in a style suitable for publication in a scientific journal. As a result repetition, of some information could not be avoided.

9. LITERATURE CITED

Allee W. C., Emerson A. E., Park O., Park T., Schmidt K. P. (1949). Principles of animal ecology. Saunders, Philadelphia.

Atlas R. M., Bartha R. (1998). Interactions among microbial populations**.** In: Atlas R. M., Bartha R. (Eds) *Microbial Ecology: Fundamentals and Applications, 4th edition*. Benjamin/Cummings Science Publishing, California, pp 85-91.

Bell A. A., Wheeler M. H. (1986). Biosynthesis and functions of fungal melanins. *Annu. Rev. Phytopathol*. **24:** 411–451.

Bemis D. A., Krahwinkel D. J., Bowman A., Mondon P., Kwon-Chung K. J. (2000). Temperature-sensitive strain of *Cryptococcus neoformans* producing hyphal elements in a feline nasal granuloma. *J. Clin Microbiol.* **38, 2:** 926-928.

Boekhout T., Theelen B., Diaz M., Fell J. W., Hop W. C. J., Abeln E. C. A., Dromer F., Meyer W. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiol.* **147:** 891-907.

Bose I., Reese A. J., Ory J. J., Janbon G., Doering T. L. (2003). A yeast under cover: the capsule of *Cryptococcus neoformans*. *Eukaryot. Cell.* **2, 4:** 655-663.

Brandt M. E., Hutwagner L. C., Klug L. A., Baughman W. S., Rimland D., Graviss E. A., Hamill R. J., Thomas C., Pappas P. G., Reingold A. L., Pinner R. W. (1996). Molecular subtype distribution of *Cryptococcus neoformans* in four areas of the United States. Cryptococcal Disease Active Surveillance Group. *J. Clin. Microbiol.* **34:** 912-917.

Brandt M. E., Pfaller M. A., Hajjeh R. A., Hamill R. J., Pappas P. G., Reingold A. L., Rimland D., arnocki D. W. for the Cryptococcal Disease Active Surveillance Group (2001). Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates in the

United States: 1992 to 1994 and 1996 to 1998. *Antimocrob. Agnets Chemother.* **45, 11:** 3065-3069.

Brueske C. H. (1986). Proteolytic activity of a clinical isolate of *Cryptococcus neoformans*. *J. Clin. Microbiol.* **23:** 631–633.

Buchanan K. L., Murphy J. W. (1998). What makes *Cryptococcus neoformans* a pathogen? *Emerg. Infect. Dis.* **4, 1:** 72-83.

Casadevall A. and Perfect J.R. (1998). *Cryptococcus neoformans*. ASM Press, Washington, DC.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready made' virulence and 'dual use' virulence factors in pathogenic environment-the *Cryptococcus neoformans* paradigm. *Curr. Opin. Microbiol.* **6:** 332-337.

Casadevall A. (2005). Fungal virulence, vertebrate endothermy, and dinosaur extinction: is there a connection? *Fungal Genet. Biol.* **42:** 98-106.

Chang Y. C.,Wickes B. L.,Miller G. F., Penoyer L. A., Kwon-Chung K. J. (2000). *Cryptococcus neoformans STE12alpha* regulates virulence but is not essential for mating. *J. Exp. Med.* **191:** 871-882.

Chang Y. C., Penoyer L. A., Kwon-Chung K. J. (2001). The second *STE12* homologue of *Cryptococcus neoformans* is MAT*a*-specific and plays an important role in virulence. *Proc. Natl. Acad. Sci. USA.* **98:** 3258–3263.

Chen F., Currie B. P., Chen L. C., Spitzer S. G., Spitzer E. D., Casadevall A. (1995). Genetic relatedness of *Cryptococcus neoformans* clinical isolates grouped with the repetitive DNA probe CNRE-1. *J. Clin. Microbiol.* **33:** 2818-2822.

Chen L. C., Blank E. S., Casadevall A. (1996). Extracellular proteinase activity of *Cryptococcus neoformans. Clin. Diagn. Lab. Immunol.* **3, 5:** 570-574.

Chrétien F., Lortholary O., Kansua I., Neuville S., Gray F., Dromer F. (2002). Pathogenesis of cerebral *Cryptococcus neoformans* infection after Fungemia. *J. Infect. Dis*. **186:** 522-530.

Clarke D. L., Woodlee G. L., McClelland C. M., Seymour T. S., Wickes B. L. (2001). The *Cryptococcus neoformans STE11alpha* gene is similar to other fungal mitogen-activated protein kinase kinase kinase (MAPKKK) genes but is mating type specific. *Mol. Microbiol.* **40:** 200-213.

Claus H. (2004). Lacasses: structure, reactions, distribution. *Micron.* **35:** 93-96.

Cox G. M., Mukherjee J., Cole G. T., Casadevall A., Perfect J. R. (2000). Urease as a virulence factor in experimental cryptococcosis. *Infect. Immun.* **68:** 443–448.

Cox G. M., McDade H. C., Chen S. C., Tucker S. C., Gottfredsson M., Wright L. C., Sorrell T. C., Leidich S. D., Casadevall A., Ghannoum M. A., Perfect J. R. (2001). Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. *Mol. Microbiol.* **39:** 166–175.

Davidson R. C., Moore T. D., Odom A. R., Heitman J. (2000). Characterization of the MFalpha pheromone of the human fungal pathogen *Cryptococcus neoformans*. *Mol. Microbiol.* **38:** 1017-1026.

Decho A.W. (1990). Microbial exopolymer secretions in ocean environments: Their role(s) in food webs and marine processes. *Oceanogr. Mar. Biol. Annu. Rev.* **28:** 73–153.

Denning D. W., Tucker R. M., Hanson L. H., Hamilton J. R., Stevens D. A. (1989). Itraconazole therapy for cryptococcal meningitis and cryptococcosis. *Arch. Intern. Med.* **149:** 2301–8.

de Silva D, Davis-Kaplan S, Fergestad J, Kaplan J. (1997). Purification and characterization of Fet3 protein, a yeast homologue of ceruloplasmin. *J. Biol. Chem.* **272, 22:** 14208-14213.

Doering T. L., Nosanchuk J. D., Roberts W. K., Casadevall A. (1999). Melanin as a potential cryptococcal defense against microbiocidal proteins. *Med. Mycol.* **37:** 175-181.

Dromer F., **Mathoulin S**., **Dupont B**., **Brugiere O**., **Letenneur L. (1996).** Comparison of the efficacy of amphotericin B and fluconazole in the treatment of cryptococcosis in human immunodeficiency virus–negative patients: retrospective analysis of 83 cases. *Clin. Infect. Dis*. **22:** 154–60.

Esser K., Meinhardt F. (1977). A common genetic control of dikaryotic and monokaryotic fruiting in the basidiomycete *Agrocybe aegerita*. *Mol. Gen. Genet.* **155:** 113-115.

Feldmesser M., Tucker S., Casadevall A. (2001). Intracellular parasitism of macrophages by *Cryptococcus neoformans. TRENDS Microbiol.* **9, 6:** 273-278.

Fell J. W., Boekhout T., Fonseca A., Scorzetti G., Statzell-Tallman A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int. J. Syst. Evol. Microbiol*. **50:** 1351–1371.

Fortes S. T., Lazéra M. S., Nishikawa M. M., Macedo R. C., Wanke B. (2001). First isolation of *Cryptococcus neoformans* var. *gattii* from a native jungle tree in the Brazilian Amazon rainforest. *Mycoses.* **44, 5:** 137-40.

Franzot S. P., Hamdan J. S., Currie B. P., Casadevall A. (1997). Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: evidence for both local genetic differences and a global clonal population structure. *J. Clin. Microbiol.* **35:** 2243-2251.

Fraser J. A., Subaran R. L., Nichols C. B., Heitman J. (2003). Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*: Implications for an outbreak on Vancouver Island, Canada. *Eukaryot. Cell*. **2, 5:** 1036-1045.

Fraser J. A., Giles S. S., Wenink E. C., Geunnes-Boyer S. G., Wright J. R., Diezmann S., Allen A., Stajichl J. E., Dietrichl F. S., Perfect J. R., Heitman J. (2005). Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature Lett.* **437, 27:** 1360-1364.

Freed E. R., Duma R. J., Shadomy H. J., Utz J. P. (1971). Meningoencephalitis due to hyphae-forming *Cryptococcus neoformans. Am. J. Clin. Pathol.* **55:** 30-33.

Garcia-Hermoso D., Janbon G., Dromer F. (1999). Epidemiological evidence for dormant *Cryptococcus neoformans* infection. *J. Clin. Microbiol.* **37:** 3204-3209.

Garcia-Rivera J., Casadevall A. (2001). Melanization of *Cryptococcus neoformans* reduces its susceptibility to the antimicrobial effects of silver nitrate. *Med. Mycol.* **39:** 353-357.

Ghannoum M. A. (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clin. Microbiol. Rev.* **13:** 122–143.

Goldman D. L., Lee S. C., Mednick A. J., Montella L., Casadevall A. (2000). Persistent *Cryptococcus neoformans* pulmonary infection in the rat is associated with intracellular parasitism, decreased inducible nitric oxide synthase expression, and altered antibody responsiveness to cryptococcal polysaccharide. *Infect. Immun.* **68, 2:** 832-838.

Goldman D. L., Khine H., Abadi J., Lindenberg D. J., Pirofski L., Njang R., Casadevall A. (2001). Serologic evidence for *Cryptococcus neoformans* infection in early childhood. *Pediatrics.* **107:** 66-73.

Guerrero A., Jain N., Goldman D. L., Fries B. C. (2006). Phenotypic switching in *Cryptococcus neoformans. Microbiol.* **152:** 3-9.

Halliday C., Bui T., Krockenberger M., Malik R., Ellis D., Carter D. (1999). Presence of α and *a* mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gatti* strains from Australia. *J. Clin. Microbiol.* **37:** 2920-2926.

Halliday C. L., Carter D. A. (2003). Clonal reproduction and limited dispersal in an environmental population of *Cryptococcus neoformans* var. *gattii* isolates from Australia. *J. Clin. Microbiol.* **41, 2:** 703-711.

Harrison T. (2000). *Cryptococcus neoformans* and cryptococcosis. *J. Infect.* **41:** 12-17.

Hill H. Z. (1992). The function of melanin or six blind people examine an elephant. *Bioessays.* **14:** 49–56.

Heitman J. (2006). Sexual Reproduction and the evolution of microbial pathogens. *Curr. Biol.* **16:** 711-725.

Hull C. M., Heitman J. (2002). Genetics of *Cryptococcus neoformans*. Annu. *Rev. Genet.* **36:** 557-615.

Husain S., Wagener M. M., Singh N. (2001). *Cryptococcus neoformans* infection in organ transplant recipients: variables influencing clinical characteristics and outcome. *Emerg. Infect. Dis.* **7:** 375-381.

Ibrahim A. S., Filler S. G., Alcouloumre M. S., Kozel T. R., Edwards J. E., Ghannoum M. A. (1995). Adherence to and damage of endothelial cells by *Cryptococcus neoformans In vitro*: Role of the capsule. *Infect. Immun.* **63, 11:** 4368-4374.

Ikeda R., Sugita T., Jacobson E. S., Shinoda T. (2003). Effects of melanin upon susceptibility of *Cryptococcus* to antifungals. *Microbial. Immunol.* **47, 4:** 271-277.

Janbon G. (2004). *Cryptococcus neoformans* capsule biosynthesis and regulation. *FEMS Yeast Res.* **4:** 765-771.

Jones B., Larsen R. A., Forthal D., Haghighat D., Bozzette S. (1991). Treatment of non-meningeal cryptococcal disease in HIV-infected persons (abstract F4). In: Proceedings of the 91st annual meeting of the American Society for Microbiology (Dallas, TX). Washington, DC: American Society for Microbiology.

Karos M., Chang Y. C., McClelland C. M., Clarke D. L., Fu J., Wickes B. L., Kwon-Chung K. J. (2000). Mapping of the *Cryptococcus neoformans MATalpha* locus: presence of mating type-specific mitogen-activated protein kinase cascade homologs. *J. Bacteriol.* **182:** 6222-6227.

Kligman A. M., Weidman F. D. (1949). Experimental studies on treatment of human torulosis. *Arch. Derm. Syph*. **60:** 726–741.

Kraus P. R., Boily M. J., Giles S. S., Stajich J. E., Allen A., Cox G. M., Dietrich F. S., Perfect J. R., Heitman J. (2004). Identification of *Cryptococcus neoformans* temperature-regulated genes with a genomic-DNA microarray. *Eukaryot. Cell.* **3, 5:** 1249-1260.

Kovacs J. A., Kovacs A. A., Polis M., Wright W. C., Gill V. J., Tuazon C. U., Gelmann E. P., Lane H. C., Longfield R., Overturf G., Macher A. M., Fauci A. S., Parrillo J. E., Bennett J. E., Masur H. (1985). Cryptococcosis in the acquired immunodeficiency syndrome. *Ann. Intern. Med.* **103:** 533–538.

Kuhlman H. W., Heckmann K. (1985). Interspecific morphogens regulating prey – predator relationships in protozoa. *Science.* **227:** 1347-1349.

Kwon-Chung K. J. (1975). A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. *Mycologia.* **61, 6:** 1197-1200.

Kwon-Chung K. J. (1976). A new species of *Filobasidiella*, the sexual state of *Cryptococcus neoformans* B and C serotypes. *Mycologia*. **68:** 943- 946.

Kwon-Chung K. J. (1980). Nuclear genotypes of spore chains of *Filobasidiella neoformans* (*Cryptococcus neoformans*). *Mycologia.* **72:** 418-422.

Kwon-Chung K. J., Polacheck I., Popkin T. J. (1982). Melanin lacking mutants of *Cryptococcus neoformans* and their virulence for mice. *J. Bacteriol.* **150:** 1414-1421.

Kwon-Chung K. J., Edman J. C., Wickes B. L. (1992). Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infect. Immun.* **60:** 602-605.

Langfelder K., Streibel M., Jahn B., Haase G., Brakhage A. A. (2003). Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genet. Biol.* **38:** 143-158.

Lazéra M. S., Pires F. D. A., Camillo-Coura L., Nishikawa M. M., Bezerra C. C. F., Trilles L. & Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *J. Med. Vet. Mycol.* **34:** 127-131.

Lengeler K. B., Wang P., Cox G. M., Perfect J. R., Heitman J. (2000). Identification of the MAT*a* mating-type locus of *Cryptococcus neoformans* reveals a serotype A MAT*a* strain thought to have been extinct. *Proc. Natl. Acad. Sci.USA.* **97, 26:** 14455–14460.

Lengeler K. B., Fox D. S., Fraser J. A., Allen A., Forrester K., Dietrich F. S., Heitman J (2002). Mating-type locus of *Cryptococcus neoformans*: a step in the evolution of sex chromosomes. *Eukaryot. Cell.* **1, 5:** 704-718.

Litvinseva A. P., Thakur R., Vilgalys R., Mitchell T. G. (2006). Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (Serotype A), including a unique population in botswana. *Genet.* **172:** 2223-2238.

Liu L., Tewari R. P., Williamson P. R. (1999). Laccase protects *Cryptococcus neoformans* from antifungal activity of alveolar macrophages. *Infect. Immun.* **67, 11:** 6034-6039.

Loftus B. J., Fung E., Roncaglia P., Rowley D., Amedeo P., Bruno D., Vamathevan J., Miranda M., Anderson I. J., Fraser J. A., Allen J. E., Bosdet I. E., Brent M. R., Chiu R., Doering T. L., Donlin M. J., D'Souza C. A., Fox D. S., Grinberg V., Fu J., Fukushima M., Haas B. J., Huang J. C., Janbon G., Jones S. J., Koo H. L., Krzywinski M. I., Kwon-Chung J. K., Lengeler K. B., Maiti R., Marra M. A., Marra R. E., Mathewson C. A., Mitchell T. G., Pertea M., Riggs F. R., Salzberg S. L., Schein J. E., Shvartsbeyn A., Shin H., Shumway M., Specht C. A., Suh B. B., Tenney A., Utterback T R., Wickes B L., Wortman J R., Wye N. H., Kronstad J. W., Lodge J. K., Heitman J., Davis R. W., Fraser C. M., Hyman R. W. (2005). The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans. Science.* **307:** 1321-1324.

Martinez L. R., Garcia-Rivera J., Casadevall A. (2001). *Cryptococcus neoformans* var. *neoformans* (Serotype D) strains are more susceptible to heat than *C. neoformans* var. *grubii* (Serotype A) strains. *J. Clin. Microbiol.* **39, 9:** 3365-3367.

Mayer A. M., Staples R. C. (2002). Laccase: new functions for and old enzymes. *Phytochem.* **60:** 551-565.

McCarthy K., Crewe-Borwn H. H., Mhlongo N., Mirza S., Brandt M., Haijeh R and the Gauteng Cryptococcal Surveillance Group (2003). The Burden of Cryptococcosis in Gauteng Province (Johannesburg), South Africa: Results of Population-based Active Surveillance, 2002-2003. Communicable Diseases Surveillance Bulletin (http://www.nicd.ac.za), 10-12.

McClelland C. M., Fu J., Woodlee G. L., Seymour T. S., Wickes B. L. (2002). Isolation and characterization of the *Cryptococcus neoformans MATa* pheromone gene. *Genet.* **160:** 935-947.

Min K. L., Kim Y. H., Kim Y. W., Jung H. S., Hah Y. C. (2001). Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis. Arch. Biochem. Biophys.* **392, 2:** 279-286.

Mondon P., Petter R., Amalfitano G., Luzzati R., Concia E., Polacheck I., Kwon-Chung K. J. (1999). Heteroresistance to fluconazole and voriconazole in *Cryptococcus neoformans. Antimicrob. Agents Chemother.* **43, 8:** 1856-1861.

Moore T. D., Edman J. C. (1993). The alpha-mating type locus of *Cryptococcus neoformans* contains a peptide pheromone gene. *Mol. Cell. Biol.* **13:** 1962-1970.

Mylonakis E., Ausubel F. M., Perfect J. R., Heitman J., Calderwood S. B. (2002). Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis. *Proc. Natl. Acad. Sci.USA.* **99, 24:** 15675-15680.

Neilson J. B., Ivey M. H., Bulmer G. S. (1978). *Cryptococcus neoformans*: pseudohyphal forms surviving culture with *Acanthamoeba polyphaga. Infect. Immun.* **20:** 262-266.

Nielsen K., Cox G. M., Wang P., Toffaletti D. L., Perfect J. R., Heitman J. (2003). Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic *a* and α isolates. *Infect. Immun.* **71, 9:** 4831-4841.

Nessa K., Gross N. T., Jarstrand C., Johansson A., Camner P. (1997). *In vivo* interaction between alveolar macrophages and *Cryptococcus neoformans*. *Mycopathologia.* **139:** 1-7.

Pappas P. G., Perfect J., Larsen R. A. (1998). Cryptococcosis in HIV-negative patients: analysis of 306 cases (abstract 101). In: 36th annual meeting of the Infectious Diseases Society of America (Denver, CO). Alexandria,VA: Infectious Diseases Society of America.

Perfect J. R. (1995). *Cryptococcus neoformans*: A sugar-coated killer with designer genes. *FEMS Immunol. Med. Microbiol.* **45; 3:** 365-404.

Perfect J. R. (2006). *Cryptococcus neoformans*: the yeast that likes it hot. *FEMS Yeast Res.* **6:** 463-468.

Powderly W. G., **Saag M. S**., **Cloud G. A**., **Robinson P**., **Meyer R. D**., **Jacobson J. M**., **Graybill J. R**., **Sugar A. M**., **McAuliffe V. J**., **Follansbee S. E**. **(1992).** A controlled trial of fluconazole or amphotericin B to prevent relapse of cryptococcal meningitis in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **326:** 793–8.

Powderly W. G. (1993). Cryptococcal meningitis and AIDS. *Clin. Infect. Dis.* **17:** 837–842.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia.* **151:** 63-69.

Rittenberg S. C. (1983). *Bdellovibrio*: Attack, penetration and growth on its prey. *ASM News*. **49:** 435-439.

Rivera J., Feldmesser M., Cammer M., Casadevall A. (1998). Organ-dependent variation of capsule thickness in *Cryptococcus neoformans* during experimental murine. *Infect. Immun.* **66, 10:** 5027-5030.

Rosas A. L, Casadevall A. (1997). Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold. *FEMS Microbiol. Lett.* **153:** 265-272.

Rosas A. L., Casadevall A. (2001). Melanin decreases the susceptibility of *Cryptococcus neoformans* to enzymatic degradation. *Mycopathologia.* **151:** 53-56.

Rozenbaum R., Rios Goncalves A. J. (1994). Clinical epidemiological study of 171 cases of cryptococcosis. *Clin. Infect. Dis.* **18:** 369–380.

Ruma-Haynes P., Brwonlee A. G., Sorrell T. C. (2000). A rapid method for detecting extracellular proteinase activity in Cryptococcus neoformans and survey of 63 isolates. *J. Med. Microbiol.* **49:** 733-737.

Saag M. S., **Cloud G. A**., **Graybill J. R**., **Sobel J. D**., **Tuazon C. U**., **Johnson P. C**., **Fessel W. J**., **Moskovitz B. L**., **Wiesinger B**., **Cosmatos D**., **Riser L**., **Thomas C**., **Hafner R**., **Dismukes W. E**. **(1999).** A comparison of itraconazole versus fluconazole as maintenance therapy for AIDS-associated cryptococcal meningitis. *Clin. Infect. Dis.* **28:** 291–6.

Saag M. S., Graybill R. J., Larsen R. A., Pappas P. G., Perfect J. R., Powderly W. G., Sobel J. D., Dismules W. E. for the Mycoses Study Group Cryptococcal Subproject (2000). Practice guidelines for the management of cryptococcal disease. *Clin. Infect. Dis.* **30:** 710–8.

Salas S. D., Bennett J. E., Kwon-Chung K. J., Perfect J. R., Williamson P. R. (1996). Effect of the laccase gene, *CNLAC1*, on virulence of *Cryptococcus neoformans. J. Exp. Med.* **184:** 377-386.

Shadomy H. J., Utz J. P. (1966). Preliminary studies on a hypha forming mutant of *Cryptococcus neoformans. Mycologia*. **58:** 383-390.

Shadomy H. J., Lurie H. I. (1971). Histopathological observations in experimental cryptococcosis causedby a hypha-producing strain of *Cryptococcus neoformans* (Cowardstrain) in mice. *Sabouraudia.* **9:** 6-9.

Shen W.C., Davidson R. C., Cox G. M., Heitman J. (2002). Pheromones stimulate mating and differentiation via paracrine and autocrine signaling in *Cryptococcus neoformans. Eukaryot. Cell.* **1:** 366–377.

Slobodkin L. B. (1968). How to be a predator. *American Zoolog.* **8**: 43-51.

Sorrell T. C., Chen S. C. A., Ruma P., Meyer W., Pfeiffer T. J., Ellis D. H., Brownlee A. G. (1996). Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. *J. Clin. Microbiol.* **34, 12:** 1253-1260.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans. Rev. Iberoam. Microbiol.* **14:** 42-43.

Steen B. R., Lian T., Zuyderuyn S., MacDonals W. K., Marra M., Jones S. J. M., Kronstad J. W. (2002). Temperature-regulated transcription in the pathogenic fungus *Cryptococcus neoformans. Genome Res.* **12:** 1386-1400.

Steenbergen J. N., Shuman H. A., Casadevall A. (2001). *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proc. Natl. Acad. Sci.USA.* **98, 26:** 15245-15250.

Steenbergen J. N., Casadevall A. (2003). The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. *Microbes Infect.* **5:** 667-675.

Taylor M., Chadwick D., Barkham T. (2002). First reported isolation of *Cryptococcus neoformans* var. *gattii* from a patient in Singapore. *J. Clin. Microbiol.* **40:** 3098-3099.

Thurston C. F. (1994). The structure and function of fungal laccases. *Microbiol.* **140:** 19–26.

Tortorano A. M., Roverselli A., Cogliati M., Brasca M. (1993). Survey of *Cryptococcus neoformans* susceptibility to antifungals in Northern Italy. (Abstract P6-7), 2nd International Conference on Cryptococcusand Cryptococcosis, Milano.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout, T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Med. Mycol.* **41:** 383-390.

Tscharke R. L., Lazéra M., Chang Y. C., Wickes B. L., Kwon-Chung K. J. (2003). Haploid fruiting in *Cryptococcus neoformans* is not mating type α-specific. *Fungal Genet. Biol.* **39:** 230-237.

Tucker S. C., Casadevall A. (2002). Replication of *Cryptococcus neoformans* in macrophages is accompanied by phagosomal permeabilization and accumulation of vesicles containing polysaccharide in the cytoplasm. *Proc. Natl. Acad. Sci. USA.* **99, 5:** 3165-70.

Valderrama B., Oliver P., Medrano-Soto A., Vazquez-Duhalt R. (2003). Evolutionary and structural diversity of fungal laccases. *Antonie van Leeuwenhoek.* **84:** 289–299.

van der Horst C. M., **Saag M. S**., **Cloud G. A**., **Hamill R. J**., **Graybill J. R**., **Sobel J. D**., **Johnson P. C**., **Tuazon C. U**., **Kerkering T**., **Moskovitz B. L**., **Powderly W. G**., **Dismukes W. E. (1997).** Treatment of cryptococcal meningitis associated with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **337:** 15–21.

Vilchez R., Shapiro R., McCurry K., Kormos R., Abu-Elmagd K., Fung J., Kusne S. (2003). Longitudinal study of cryptococcosis in adult solid-organ transplant recipients.*Transpl. Int.* **16:** 336-340.

Viviani M. A. (1996). Cryptococcal meningitis: Diagnosis and treatment. *Int. J. Antimicrob. Agents.* **6:** 169-173.

Wang Y., Aisen P., Casadevall A. (1996). Melanin, melanin "ghosts," and melanin composition in *Cryptococcus neoformans. Infect. Immun.* **64, 7:** 2420-2424.

Wang P., Heitman J. (1999). Signal transduction cascades regulating mating, filamentation and virulence in *Cryptococcus neoformans*. *Curr. Opin. Microbiol.* **2:** 358-362.

Wang P., Perfect J. R., Heitman J. (2000). The G-protein beta subunit *GPB1* is required for mating and haploid fruiting in *Cryptococcus neoformans. Mol. Cell. Biol.* **20:** 352-362.

Wang P., Nichols C. B., Lengeler K. B., Cardenas M. E., Cox G. M., Perfect J. R., Heitman J. (2002). Mating-type-specific and nonspecific PAK kinases play shared and divergent roles in *Cryptococcus neoformans*. *Eukaryot. Cell.* **1, 2:** 257-272.

Wickes B. L., Mayorga M. E., Edman U., Edman J. C. (1996). Dimorphism and haploid fruiting in *Cryptococcus neoformans*: Association with the α-mating type. *Microbiol.* **93:** 7327-7331.

Wickes B. L., Edman U., Edman J. C. (1997). The *Cryptococcus neoformans STE12alpha* gene: a putative *Saccharomyces cerevisiae STE12* homologue that is mating type specific. *Mol. Microbiol.* **26:** 951-960.

Wickes B. L. (2002). The role of mating type and morphology in *Cryptococcus neoformans* pathogenesis. *Int. J. Med. Microbiol.* **292:** 313-329.

Williamson J. D., Silverman J. F., Mallak C. T., Christie J. D. (1996). Atypical cytomorphologic appearance of *Cryptococcus neoformans*: a report of five cases. *Acta Cytol.* **40:** 363-370.

Williamson P. R. (1997). Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Front. Bioscience* **2:** 99-107.

Xu J., Ali R. Y., Gregory D. A., Amick D., Lambert S. E., Yoell H. J., Vilgalys R. J., Mitchell T. G. (2000). Uniparental mitochondrial transmission in sexual crosses in *Cryptococcus neoformans. Curr. Microbiol.* **40:** 269–273.

Yue C., Cavallo L. M., Alspaugh J. A., Wang P., Cox G. M., Perfect J. R., Heitman J. (1999). The *STE12alpha* homolog is requiredfor haploid filamentation but largely dispensable for mating and virulence in *Cryptococcus neoformans*. *Genet.* **153:** 1601-1615.

Zhang S., Varma A., Williamson P. R. (1999). The yeast *Cryptococcus neoformans* uses ‘mammalian’ enhancer sites in the regulation of the virulence gene, *CNLAC1*. *Gene.* **227:** 231-240.

Zhu X., Gibbons J., Garcia-Rivera J., Casadevall A., Williamson P. R. (2001). Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. *Infect. Immun.* **69, 9:** 5589-5596.

Zhu X., Williamson P. R. (2004). Role of laccase in the biology and virulence of *Cryptococcus neoformans. FEMS Yeast Res.* **5:** 1-10.

Zhu X., Gibbon J., Zhang S., Williamson P. (2004). Copper mediated reversal of defective laccase in a *vph1* avirulent mutant of *Cryptococcus neoformans*. *Mol. Microbiol.* **47:** 1007–1014.

Chapter 2

Isolation, Identification and Characterization of *Cryptococcus neoformans* Strains Originating from Three Provinces of South Africa

Isolation, Identification and Characterization of *Cryptococcus neoformans* Strains Originating from Three Provinces of South Africa

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1. ABSTRACT

A total of four environmental *C. neoformans* var. *grubii* strains, isolated from soil in the North West province of South Africa, and 32 clinical *C. neoformans* strains, originating from the Gauteng and Western Cape provinces of South Africa, were identified and characterized. Strains were identified by sequencing the internal transcribed spacer (ITS) region of the ribosomal gene cluster, while sero and mating type were confirmed using polymerase chain reaction (PCR) primers. The genotype of each strain was determined by employing three PCR based typing techniques, namely PCR fingerprinting using the mini-satellite M13, micro-satellite $(GACA)_4$ and random amplified polymorphic DNA (RAPDS), as well as restriction fragment length polymorphism (RFLP) analysis of the phopholipase B1 gene. A total of 97 % of the strains were identified as *C. neoformans* var. *grubii* (serotype A), while only one strain was identified as *C. neoformans* var. *neoformans* (serotype D). All strains were found to be MATα and haploid. The majority of strains grouped into genotype VNI (75.6 %), seven strains belong to genotype VNII (21.2 %) while only one strain represented genotype VNIV (3 %). All results are in line with global and local trends observed for *C. neoformans*.

2. INTRODUCTION

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic fungal pathogen responsible for causing meningitis predominantly in immuno-compromised individuals (Casadevall *et al*., 2003; Franzot *et al*., 1998; Mitchell and Perfect, 1995), particularly those suffering from human immuno virus (HIV) and acquired immuno-deficiency syndrome (AIDS). The incidence of this infection, also known as cryptococcosis, among these individuals is estimated at approximately 10 % (Chuck and Sande, 1989). Although the

incidence of infection is less in organ transplant patients (5 %), the mortality rates are estimated at approximately 50 % of all cases (Vilchez *et al*., 2003; Husain *et al*., 2001). This basidiomycetous yeast (Boekhout *et al*., 1997) is usually characterized by the production of melanin, resulting in typical brown colony pigmentation when cultured on differential media, particularly Niger seed (*Guizotia abyssinica*) agar (Yarrow, 1998). Two main varieties are known, *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A), with a hybrid between these varieties, *C. neoformans* (serotype AD) also identified (Boekhout *et al*., 2001). Unlike the perceived localization of *Cryptococcus gattii* (Sanfelice) Vuilemin, *C. neoformans* is well known for its worldwide distribution (Sorrell *et al*., 1997) and has been isolated from a number of environmental sources including soil, avian guano contaminated soil, avian guano, fur trees, almond tress, eucalyptus trees, woody debris and decaying wood (Trilles *et al*., 2003; Halliday *et al*., 1999; Sorrell *et al*., 1997; Lazéra *et al*., 1996).

Kwon-Chung was able to identify the sexual state of *C. neoformans* and *C. gattii*, namely *Filobasidiella neoformans* and *Filobasidiella bacillisporus*, respectively (Kwon-Chung, 1976; Kwon-Chung, 1975). Although occurring primarily as unicellular yeast cells that reproduce asexually by budding (Boekhout *et al*., 1997), *C. neoformans* is capable of dimorphic transitions resulting in the development of hyphae, with fused clamp connections capable of forming basidia and ultimately basidiospores (Kwon-Chung, 1975). These basidiospores are the proposed infectious propagules that are able enter the human system via the lungs (Ellis and Pfeiffer, 1990). Genetic analysis revealed that *C. neoformans* possesses a bipolar mating system with two distinct mating types, namely mating type *a* (MAT*a*) and mating type alpha (MATα), determined by a single locus (McClelland *et al*., 2002; Kwon-Chung, 1976). Interestingly, MATα appears to be more prevalent than its counterpart with regards to both clinical and environmental sources and this occurrence has led to speculation that MATα cells are more virulent and better suited for survival (Kwon-Chung *et al*., 1992). However, experimentation involving several animal models has shown no difference in virulence between mating types of *C. neoformans* var. *grubii* (serotype A) (Nielsen *et al*., 2003). It has therefore been hypothesized that mating type may not play a pivotal role in the virulence of all the serotypes and other factors need to be considered when examining the mating type bias. These factors include haploid fruiting (Wickes *et al*., 1996) and the possibility of lethal mutations (Kwon-Chung *et al*., 1992).

Coupled to the discovery of the sexual states of *C. neoformans* and *C. gattii*, the advancement in molecular science has meant that these yeast pathogens now serve as models

with regards to pathogenicity within the basidiomycetous fungi. Several molecular typing methods were developed in order to analyze both clinical and environmental isolates of *C. neoformans*. These typing techniques include Southern blot hybridization with DNA probes (Varma *et al*., 1995), karyotyping (Perfect *et al*., 1993), polymerase chain reaction (PCR) fingerprinting (Meyer *et al*., 1999; Meyer *et al*., 1993), random amplified polymorphic DNA (RAPDs) (Meyer *et al*., 1999, Chen *et al*., 1997; Sorrell *et al*., 1996), restriction fragment length polymorphism (RFLP) (Latouche *et al*., 2003) and sequence analysis (Franzot *et al*., 1997). Using these techniques *C. neoformans* strains were grouped into four major genotypic groups, namely VNI, VNII, VNIII and VNIV. Further analysis of these genotypes using micro and mini-satellite PCR fingerprinting revealed significant intra-genotype diversity among genotype VNI strains (Meyer *et al*., 1999). Interestingly, in the case of *C. neoformans*, the genotypes appear to correspond with the various serotypes. *C. neoformans* var. *grubii*, serotype A, is designated to genotypes VNI and VNII, *C. neoformans* var. *neoformans* is designated to genotype VNIV, while the hybrid species, serotype AD, is designated to genotype VNIII. A recent study conducted by Litvintseva and co-workers (2006) revealed the existence of a possible fifth genotypic group, designated group VNB, although this group appears to be indigenous to Botswana.

From a global medical perspective, *C. neoformans* var. *grubii* (Serotype A, MATα) is responsible for approximately 90 % of all cryptococcosis infections (Mitchell and Perfect, 1995). Although representatives of this variant can be grouped into two different genotypes, namely VNI and VNII, the genotype VNI appears to dominant having a worldwide distribution (Meyer *et al*., 1999). This genotype has been isolated from 78 % of global AIDS related cryptococcal infections. Similarly, it was isolated from 57 % and 88 % of AIDS related cryptococcal infections in the province of Gauteng and Kwa-Zulu Natal, South Africa, respectively. In the latter study, isolates originating from two of the provinces of South Africa were analyzed. We were interested whether genotype VNI is also dominant among cases of AIDS related cryptococcal infections originating from the Western Cape Province of South Africa.

With the above as background, the objective of this preliminary study was to obtain an indication of the prevailing *C. neoformans* genotypes occurring within the HIV positive and AIDS populations of South Africa and to obtain an indication of its distribution within the environment, particularly within the Western Cape Province.

3. MATERIALS AND METHODS

3.1. Strains and culture conditions

Cryptococcus neoformans var. *neoformans* CBS 132, CBS 10079; *C. neoformans* var. *grubii* CBS10515 (H99), CBS 10085, CBS 10084, CBS 9172; *C. neoformans* CBS 10080 and *C. gattii* CBS 10101, CBS 10082 were obtained from the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. The environmental *C. neoformans* var. *grubii* isolates, CBS 10571, CBS 10572, CBS 10573, CBS 10574, and the clinical *C. gattii* strain, CBS 10575 obtained during this study, have all been deposited into the culture collection of the CBS, Utrecht, The Netherlands. Clinical *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* strains were obtained from the culture collection of the Medical Research Council (MRC), PROMEC Unit, Tygerberg, South Africa (see Appendix A). A total of three *C. neoformans* var. *grubii* strains were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa (see Appendix A). The reference strains *C. laurentii* ABO-1A, *C. podzolicus* ABO-5A and *Saccharomyces cerevisiae* ABO-SC1 were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa. All strains were maintained by periodic transfer to yeast peptone glucose (YPG, pH 5.5) agar supplemented with 200 mg/L chloramphenicol (Sigma, Cat no: CO378-25G) (Yarrow, 1998) and incubated at 22 °C.

3.2. Sampling

Soil, avian guano contaminated soil, avian guano, vegetative debris and plant material were sampled throughout South Africa at various times of the year and from various biomes in order to isolate *C. neoformans* from environmental sources. Details with regards to the samples' origin, composition as well as patient information, from whom the clinical strains were originally isolated, are listed in Appendix B.

3.3. Isolation from environmental sources

Serial dilutions of the various samples were prepared in physiological saline, spread plated onto Niger seed agar supplemented with 100 mg/L biphenyl (Sigma, Cat no: B6890) and 200 mg/L chloramphenicol (Sigma, Cat no: CO378-25G) (Yarrow, 1998); whereafter plates were incubated at 26 °C, 30 °C and 37 °C for 10 days. Plates were inoculated in triplicate and observed daily for the development of pigmented colonies characteristic of

C. neoformans and *C. gattii*.

3.4. Preliminary Identification

3.4.1. Growth on Differential Medium

All strains were inoculated onto Niger seed agar, supplemented with 100 mg/L biphenyl (Sigma, Cat no: B6890) and 200 mg/L chloramphenicol (Sigma, Cat no: CO378-25G) (Yarrow, 1998), and incubated at 37 °C for a total of 10 days. Plates were observed daily for the development of brown pigmented colonies characteristic of *C. neoformans* and *C. gattii*. Pigmented colonies were further cultured on YPG agar (pH 5.5) (Yarrow, 1998) supplemented with 200 mg/L chloramphenicol (Sigma, Cat no: CO378-25G).

Strains *C. neoformans* var. *grubii* H99 and *C. neoformans* var. *neoformans* CBS 132 were included as positive controls in the experimentation, while *S. cerevisiae* ABO-SC1 served as a negative control.

All strains tentatively identified as either *C. neoformans* or C. gattii were subsequently inoculated onto L-canavacine-glycine-bromothymol blue (CGB) agar and incubated at 22 °C for 3 days (Kwon-Chung *et al.*, 1982). Plates were observed daily for the development of a cobalt blue appearance characteristic of serotypes B and C. Positive controls included *C. gattii* CBS 10101 and *C. gattii* CBS 10082, while *C. neoformans* var. *grubii* H99 and *C. neoformans* var. *neoformans* CBS 132 served as negative controls.

3.5. Molecular Identification

3.5.1. Genomic DNA extraction

Strains were transferred from YPG (pH 5.5) agar plates (Yarrow, 1998) supplemented with 200 mg/L chloramphenicol (Sigma, Cat no: CO378-25G) to test tubes (15 mm in diameter and 150 mm in length) containing 10 mL yeast malt broth (YM, pH 5.0) and incubated at 30 °C for 18 hours on a tissue culture roll drum (10 rpm). A volume of 2 mL culture was extracted and centrifuged (Biofuge fresco, Heraeus Instruments) in 2 mL Eppendorf tubes (Seabreeze, Cape Town) for 5 min at 13 793 RCF. Pelleted cells were re-suspended in 500 µL DNA lysis buffer containing 20 % (v/v) Tris-HCl (1 M, pH 8.0); 10 % (v/v) EDTA (0.5 M); 5 % (v/v) SDS 20 % and 1300 µL sterile distilled water. Acid washed glass beads (Sigma, Cat no: G4649-100G) were added and the tubes were vortexed for 4 min (Vortex Genie 2; set at 8) and placed on ice for 5 min. A total of 275 µL ammonium acetate (7 M) was added and the tubes were incubated at 65 °C (5 min) and again placed on ice for 5 min. A volume of 500 µL chloroform was added and the tubes were vortexed and centrifuged

(Biofuge fresco, Heraues Instruments) for 5 min at 13 793 RCF (4 °C). The supernatant was removed and precipitated with 1 volume isopropanol at 22 °C for 5 min. The precipitate was centrifuged (Biofuge fresco, Heraues Instruments) for 5 min at 13 793 RCF (4 °C), and the pellet washed with 70 % (v/v) alcohol. The washed pellet was dried and dissolved in 50 µL TE buffer containing 0.5 % (v/v) Rnase A (Roche, USA, Cat no: 109169) and incubated at 37 °C for 20 min. DNA was stored at a temperature of -20 °C.

3.5.2. Analysis of the internal transcribed spacer (ITS) region

Amplification of the internal transcribed spacer (ITS) region of the ribosomal gene cluster was done using the universal primers, ITS1/5 and ITS4 as described by White and co-workers (1990), yielding a product of approximately 600 base pairs. Polymerase chain reaction (PCR) was performed in 25 µL reaction volumes with final concentrations of 10 ng DNA, 2.5 mM magnesium chloride ($MgCl_2$), 0.4 mM deoxynucleotide triphosphates (dNTPs), 0.2 µM of each primer and 0.5 U generic Taq. Amplification was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400) for 35 cycles as follows: initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 4 min. Amplified products were separated in an agarose gel (0.8 %, w/v) and visualized by ethidium bromide staining according to standard methods (Chory and Pollard, 1999).

PCR products were purified with Nucleospin® Extract II (Macherey-Nagel, Cat no: 740 609.50) chromatography columns. Sequences representing the ITS region of the ribosomal DNA (rDNA) from the strains were obtained using an ABI PRISM (model 3100) genetic sequencer. The forward and reverse sequences were aligned with DNAMAN for WINDOWS Version 4.13 (Lynnon Biosoft). The yeast strains were identified by comparing the sequencing results with known sequences using the BLAST program (www.ncbi.nlm.nih.gov/blast).

3.6. Molecular Characterization

3.6.1. Serotype determination using PCR specific primers

The serotype of each strain was determined by a modification of the method described by Lengeler and co-workers (2000) using the primer combinations JOHE5169 / JOHE5170 (Ste20*a*A); JOHE3067 / JOHE30687 (Ste20*a*D); JOHE2472 / JOHE2578 (Ste20αA) and JOHE3069 / JOHE3070 (Ste20αD). PCR was performed in 25 µL reactions containing 10 ng

DNA; 3 mM $MgCl_2$; 0.4 mM dNTPs; 0.1 μM of each primer and 1 U Taq (5 U/μL, Fermentas, Cat no: EP0402). Amplification was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400) as previously described (Lengeler *et al*., 2000). Amplified products were separated in an agarose gel (1.0 %, w/v) and visualized by ethidium bromide staining according to standard methods (Chory and Pollard, 1999).

3.6.2. Mating type determination using PCR specific primers

The mating type of each strain was determined by a modification of the method described by Chaturvedi and co-workers (2000). PCR was performed in 25 μL reaction volumes with final concentrations of 10 ng DNA, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.2 μM of each primer and 0.5 U generic Taq. Amplification was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400) for 30 cycles as follows: initial denaturation at 95 °C for 3 min, denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 1 min. The strains *C. neoformans* var. *grubii* CBS 9712 (MAT*a*) and *C. neoformans* var. *grubii* H99 (MATα) served as positive and negative controls with regard to each set of PCR primers.

Amplification products were separated in an agarose gel (1.0 %, w/v) and visualized by ethidium bromide staining according to standard methods (Chory and Pollard, 1999).

3.6.3. Genotyping

Restriction fragment length polymorphism (RFLP) of the Phospholipase gene (*PBL1*).
Analysis of *PLB1* was done by means of PCR-RFLP. PCR Amplification of *PLB1* was done using primers described by Latouche and co-workers (2003) in 25 μL reactions containing 10 ng DNA; 3 mM $MgCl_2$; 0.4 mM dNTPs; 0.1 μM of each primer and 1 U Taq (5 U/μL, Fermentas, Cat no: EP0402) using a GeneAmp PCR System (Applied Biosystems, model 2400).

A total of 10 μL PCR product was digested with 2 μL Tango buffer (10x, Fermentas, Cat no: BY5), 0.5 μL AvaI enzyme (10 U/μL; Fermentas; Cat no: ER0381) and sterile distilled water to a final volume of 20 μL in 2 ml Eppendorf tubes (Seabreeze). Tubes were incubated at 37 °C for a maximum of 1 hour.

Amplification products and restriction digest fragments were separated in an agarose gel (0.8 %, w/v and 3.0 % w/v, respectively) and visualized by ethidium bromide staining according to standard methods. The genotypes (VNI – VNIV) were assigned by comparison

to representatives of the four major genotypes (Table 1) loaded on each gel. (Chory and Pollard, 1999; Latouche *et al*., 2003).

PCR fingerprinting using the micro-satellite $(GACA)_4$ PCR fingerprinting using the micro-satellite $(GACA)_4$ was done by a modification of the method described by Cogliati and co-workers (2001). PCR was performed as previously described.

Amplification products were separated in an agarose gel (3.0 %, w/v) at 20 V for 18 hours and visualized by ethidium bromide staining according to standard methods (Chory and Pollard, 1999). The genotypes (VNI – VNIV) were assigned by comparison to representatives of the four major genotypes (Table 1) loaded on each gel.

PCR fingerprinting using the mini-satellite M13. PCR fingerprinting using the mini-satellite specific core sequence of the wild type phage M13 was done by a modification of the method described by Meyer and co-workers (2003). PCR was performed as previously described. Amplification products were separated in an agarose gel as previously described.

Random Amplified Polymorphic DNA (RAPD). RAPD analysis was done according to a modified method originally described by Meyer and co-workers (1999). PCR was performed as previously described. Amplification products were separated in an agarose gel as previously described.

Table 1 Reference strains of *C. neoformans* used to determine the genotypes of unknown strains representing this species.

Reference Strain	Serotype	Mating Type	Genotype
C. neoformans var. *grubii* CBS 10085	A	MATα	VNI
C. neoformans var. *grubii* CBS 10084	A	MATα	VNII
C. neoformans (hybrid) CBS 10080	AD	MAT*a* - MATα	VNIII
C. neoformans var. *neoformans* CBS 10079	D	MATα	VNIV

4. RESULTS AND DISCUSSION

4.1. Isolation of *C. neoformans* from environmental sources

Despite attempts to isolate *C. neoformans* from a large variety and number of environmental sources (see Appendix B), a total of only four isolates was obtained. None were isolated from environmental sources in the Western Cape Province, South Africa. All originated from soil samples containing vegetative debris taken in the Wolmaranstad area within the North West Province of South Africa. The area is classified as grassland vegetation, has a high average rainfall of 1001-1500 mm (p.a.) and an average temperature of 17°C. The soil type and texture is classified as sandy loam to sandy clay loam (Soil Classification Working Group, 1991). All the environmental *C. neoformans* var. *grubii* isolates, CBS 10571, CBS 10572, CBS 10573, CBS 10574, have been deposited into the fungal culture collection of the CBS, Utrecht, The Netherlands (Table 2).

Possible reasons for the low isolation frequency may be attributed to the sampling technique, where single samples as opposed to composite samples were taken. In addition, very few swabs of the area surrounding the sampling site were done. However, despite these shortcomings, the low isolation frequency of *C. neoformans* from the environmental samples screened during our study may actually be a result of the infrequent occurrence of the pathogen within the environment. Granados and Castaneda (2006) evaluated the natural occurrence of *C. neoformans* and *C. gattii* over a 12 year period within the Colombian environment and achieved an isolation frequency of only 2.63 %, highlighting the limited occurrence of *C. neoformans* and *C. gattii* within that envionment.

Alternatively, the isolation medium itself may have also contributed to the low isolation frequency. While Niger seed agar is the preferred isolation medium within a clinical setting where cryptococcal numbers are high relative to other fungi, the medium does not appear to be ideal for the isolation of *C. neoformans* from the environment where numerous other fungi also occur. Our experience was that despite the addition of biphenyl, Niger seed agar did not inhibit growth of filamentous fungi that spread rapidly over the media limiting the growth of slower growing yeast species. Furthemore, the ability to produce brown colonies was recently found not to be a unique characteristic of *C. neoformans*. Okoli and co-workers (2007) have shown that the newly isolated species *Cryptotrichosporon anacardii* is capable of producing pigmented colonies when cultured on Niger seed agar. Interestingly, studies of species related to *C. neoformans*, namely *C. laurentii and C. podzolicus*, revealed that these cosmopolitan soil yeasts produce a laccase enzyme similar to the one produced by

C. neoformans, that is also capable of melanin synthesis and would therefore produce a positive result when cultured on Niger seed agar (Ikeda *et al*., 2002; Petter *et al*., 2001). Similar to *C. neoformans*, *C. laurentii* is also capable of growth at 37 °C and produces a capsule (Filion *et al*., 2006). A number of false positive results were obtained during our own isolation programme where the isolates were later identified as representatives of the ascomycetous genus *Pichia* (data not shown). Other yeasts therefore could show similar colony characteristics to *C. neoformans* when cultured on Niger seed agar. However, reasons for the infrequent occurrence of *C. neoformans* in nature remains unclear and would therefore require further investigation.

4.2. Preliminary identification

4.2.1. Growth on Differential Media

All presumptive *C. neoformans* and *C. gattii* strains were cultured on Niger seed agar at 37 °C for 10 days (Fig 1). Only one clinical strain did not produce the characteristic brown pigmentation of these pathogenic yeast species, namely strain ABO-C9, and was later identified by means of ITS sequencing to be a representative of the species *Candida albicans.*

CGB agar is generally used in order to distinguish *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* from *C. gattii* (Kwon-Chung *et al*., 1982). *C. gattii* is capable of hydrolyzing the glycine within the CGB agar, resulting in an increase in pH changing the pigmentation of medium from yellow to cobalt blue. All strains were cultured on CGB agar at 25 °C for 3 days. Only one clinical strain demonstrated a positive result, namely *C. gattii* CBS 10575 (Fig 2). The identity of this strain was later confirmed by Dr Ferry Hagen of the CBS as *C. gattii* belonging to the VGI genotype (AFLP 4). The remaining isolates tested negative (Fig 3), however some produced a green colouration towards the initial inoculum and this has been attributed to a heavy inoculum (Kwon-Chung *et al*., 1982).

4.3. Molecular identification

Molecular identification was done by sequencing the internal transcribed spacer (ITS) region of each strain and comparing the results with known sequences using the BLAST program (www.ncbi.nlm.nih.gov/blast) (see Appendix C). All but one clinical strain, namely MRC 8856, was identified as being *C. neoformans* var. *grubii* (Table 2). These results are in accordance with previous studies and stated literature that serotype A is dominant with regards to both clinical and environmental isolates. As a result, *C. neoformans* var. *grubii* is

regarded as being more virulent when compared to its counterpart *C. neoformans* var. *neoformans.*

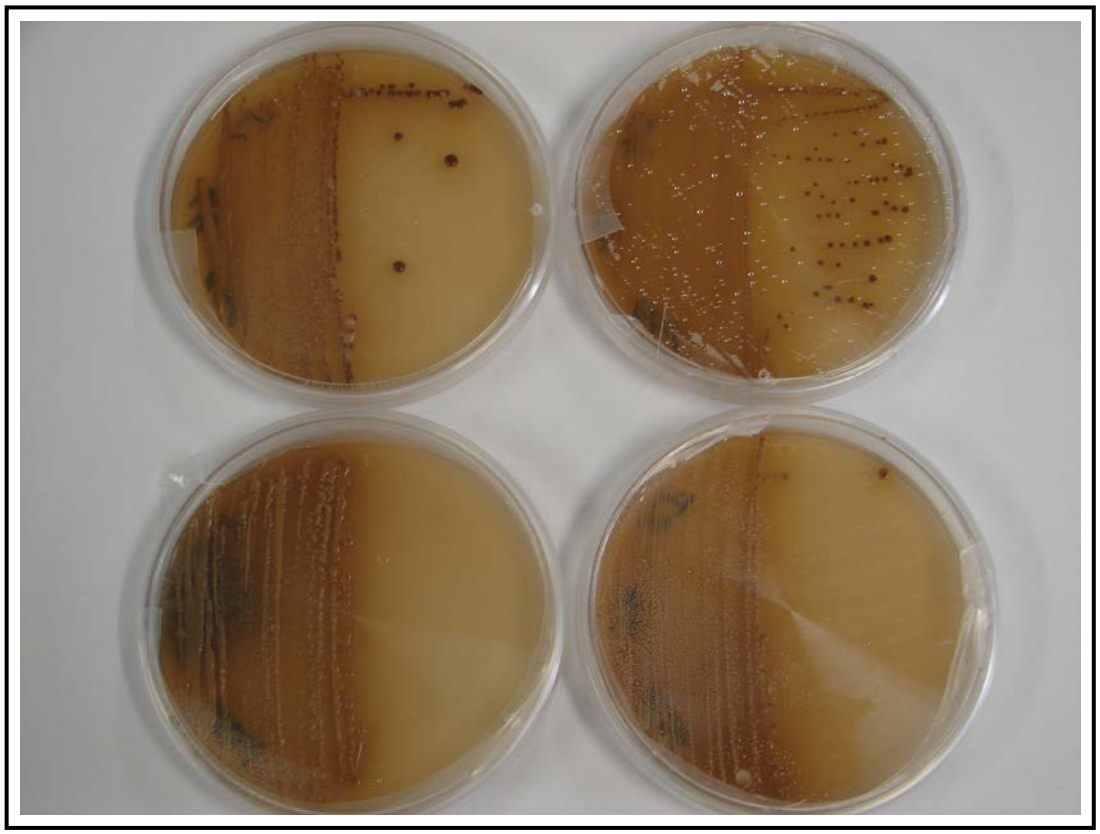

Figure 1 The environmental strains *C. neoformans* var. *grubii* CBS 10571 (top left), CBS 10572 (top right), CBS 10573 (bottom left) and CBS 10574 (bottom right) all produce the characteristic brown colony pigmentation when cultured on Niger seed agar.

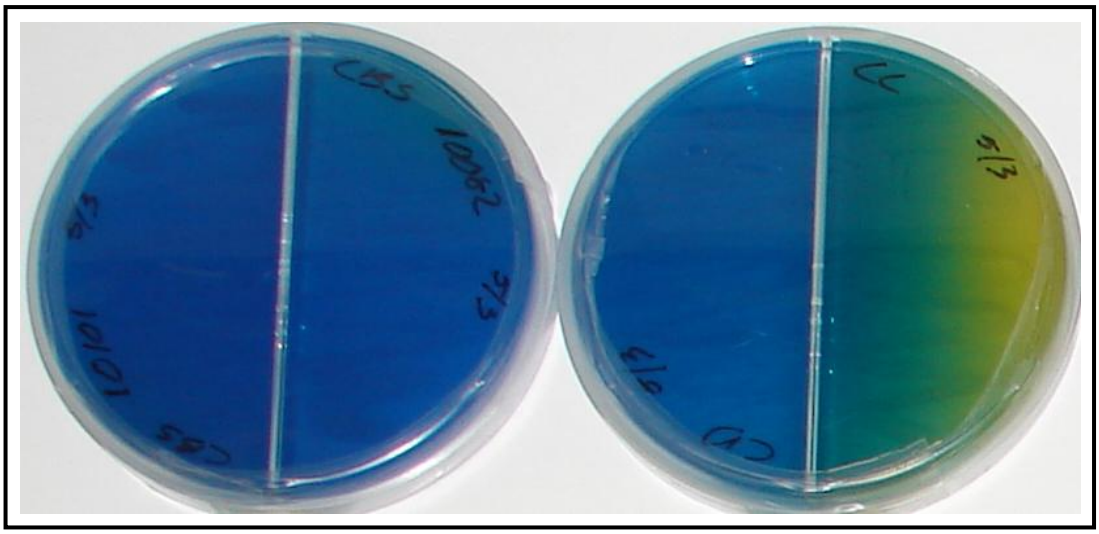

Figure 2 One *C. neoformans* strain tested positive when cultured on CGB agar producing the characteristic cobalt blue colour characteristic of serotype B and C. The inoculated strains from left to right are as follows: *C. gattii* CBS 10101, *C. gattii* CBS 10082, *C. gattii* CBS 10575 (CD) and *C. neoformans* var. *grubii* MRC 8880 (CC).

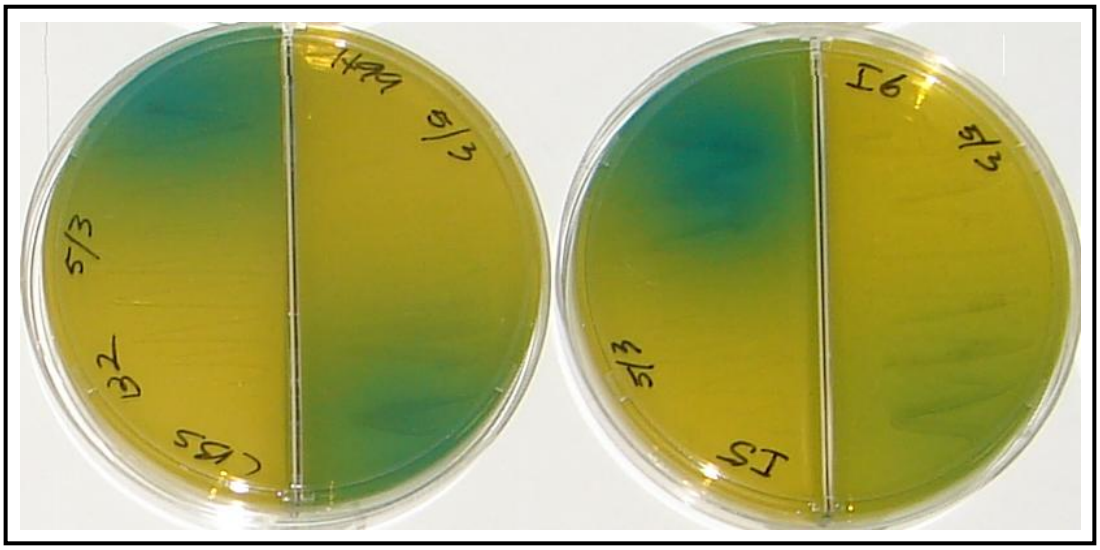

Figure 3 The majority of *C. neoformans* strains tested negative when cultured on CGB agar, however some discolouration occurred due to heavy inoculum. The inoculated strains from left to right are as follows: *C. neoformans* var. *neoformans* CBS 132, *C. neoformans* var. *grubii* H99, *C. neoformans* var. *grubii* CBS 10574 (I5), *C. neoformans* var. *grubii* CBS 10573 (I6).

4.4. Molecular Characterization

4.4.1. Serotype and mating type determination using PCR specific primers

The sero and mating type of each strain was determined using the primers described by Lengeler and co-workers (2000). Although these primers are known to display weak cross reactivity, all strains were found to be mating type α (MATα) and all but one clinical isolate, namely *C. neoformans* var. *neoformans* MRC 8856, was found to be serotype A (Table 2). The mating type of each strain was confirmed by amplifying the MAT*a* and MATα pheromone genes (*MFa* and *MF*α) (Chaturvedi *et al*., 2000). Amplification of all the strains resulted in a product of approximately 100 base pairs in length and were designated as mating type α (MATα) (Table 2).

Both the serotype and mating type results are in accordance with literature. Epidemiological studies have revealed that serotype A (*C. neoformans* var. *grubii*) is dominant with regards to both environmental and clinical isolates. In Brazil, *C. neoformans* var. *grubii* accounts for an estimated average of 83 % of all cryptococcosis cases (Barreto de Oliveira *et al*., 2004; Rozenbaum *et al*., 1992); 80 % in Canada (Kwon-Chung and Bennett, 1984); 80 % in France (Dromer *et al*., 1996); 62 % in Germany (Mitchell and Perfect, 1995); 87 % in India (Padhye *et al*., 1993); an average of 42 % in Italy (Totorano *et al*., 1997); 90 % in Taiwan (Hsu *et al*., 1994); an average of 94 % in Thailand (Poonwan *et al*., 1997); 95 % in Japan (Kohno *et al*., 1994); 75 % in the United States (Kwon-Chung and Bennett, 1984) and 63 % in Venezuela (Villanueva *et al*., 1989). Perhaps the only exception of this trend exists within northern European countries where *C. neoformans* var. *neoformans* (serotype D) displays a greater prevalence (Martinez *et al*., 2001; Dromer *et al*., 1996).

Interestingly, this observation has been attributed to the more temperate climate of these northern European countries. Martinez and co-workers (2001) were able to demonstrate that serotype D was on average more susceptible to heat killing than serotype A. On average, at temperatures of 41 ºC and higher, *C. neoformans* var. *grubii* (serotype A) displays greater thermal tolerance than *C. neoformans* var. *neoformans* (serotype D). A second case study involving the simultaneous infection by *C. neoformans* and malaria, noted that during relapses of malaria, *C. neoformans* cells were cleared from the spinal fluid (Kligman *et al*., 1949). Researchers have speculated that the fever induced by the malaria may have resulted in the elimination of *C. neoformans* cells. This thermo-intolerance displayed by *C. neoformans* var. *neoformans* could account for its higher prevalence in European countries with temperate climates, as well as the high predilection seen for skin tissue that is regarded as having a cooler overall temperature (Martinez *et al*., 2001; Dromer *et al*., 1996).

A similar dominance is seen with regards to the mating type of *C. neoformans*. Mating type alpha (MATα) is once again prevalent in both clinical and environmental settings with ratios being as high as 30:1 and 40:1 respectively (Halliday *et al*., 1999) although rare cases of even distribution between mating types have been reported. In 2006, Litvintseva and co-workers reported an unusually high proportion of fertile *C. neoformans* MAT*a* isolates in Botswana (Litvintseva *et al*., 2006). A closely related species to *C. neoformans*, *C. gattii,* demonstrates the same dominance with regards to uneven mating type distribution, however cases of even ratios have also been reported. Halliday and co-workers (1999) reported the isolation of *C. gattii* with ratios of approximately 1:1 with regards to mating type.

This significant global bias suggested that MATα is more virulent than its mating partner and perhaps better suited for survival. Using congenic strains of serotype D, Kwon-Chung and co-workers (1992) were able to show by using the murine tail vein injection model that MATα was indeed more virulent than MAT*a*. Until recently, the same conclusion could not be drawn with regards to serotype A as a MAT*a* isolate had not yet been identified and was thought to be extinct (Lengeler *et al*., 2000). The discovery of a serotype A, MAT*a* environmental strain in Italy led to the production of congenic serotype A strains (Viviani *et al*., 2001). Interestingly, virulence testing in both the murine inhalation model and rabbit immuno-suppressed infection showed no difference in virulence between the two mating types (Nielsen *et al*., 2003) indicating that unlike *C. neoformans* var. *neoformans* (serotype D), mating type may not play a vital role in the virulence of *C. neoformans* var. *grubii*.

Several alternative theories have been presented to explain the overwhelming dominance of MATα in both clinical and environmental settings. With regards to clinical isolations, the sheer exposure frequency of humans to a *C. neoformans* MATα source may account for its dominance (McClelland *et al*., 2002). Goldman and co-workers (2001) were able to show that from the age of two years, children within an urban setting had already acquired anti-bodies towards *C. neoformans*, regardless of their immune status, thereby emphasizing the ubiquitous nature of this pathogen. It was hypothesized that the dominance of MATα cells within the environment may be a result of vigorous monokaryotic fruiting displayed by this mating type under stress conditions (Wickes *et al*., 1996). Wickes and co-workers (1996) also noted that during monokaryotic fruiting, a greater number of basidiospores are produced when compared to dikaryotic fruiting and that basidiospore production was constant, not intermittent. These results would simply suggest that a greater quantity of MATα basidiospores are produced, increasing the frequency of MATα within nature. However, Tscharke and co-workers (2003) were able to show that MAT*a* cells were

also capable of monokaryotic fruiting under standard laboratory conditions illustrating yet again that a combination of factors are responsible for the mating type bias seen in *C. neoformans*.

Interestingly, same sex mating of *C. gattii* MATα has been observed amongst strains isolated from the Vancouver Island outbreak (Fraser *et al*., 2005). Genotypic analysis of these *C. gattii* strains revealed evidence of recombination; however, the offspring appeared to have descended from two MATα parents. Although the same phenomena has not been reported for *C. neoformans*, it may provide yet another alternative theory to the mating type bias observed in populations of both these pathogenic yeast species.

4.4.2. Genotyping

The genotypes of the *C. neoformans* strains were determined using a series of current techniques including RFLP analysis of the phospholipase gene (Fig 4), micro and mini-satellite PCR fingerprinting (Fig 5 to 6) as well as RAPD analysis (data not shown). All strains were classified into one of four major genotypes (Table 2) by comparing banding patterns to the four reference strains (Table 1). Four different genotyping techniques were used to ensure the accurate grouping of the strains, particularly with regards to genotypes VNI and VNII that produce similar banding patterns and are known to be prevalent in the provinces of Gauteng and Kwa-Zulu Natal, South Africa (Meyer *et al*., 1999).

We found that the majority of strains originating from the Gauteng North West and Western Cape provinces of South Africa, represented genotype VNI (75.6 %), seven strains belong to genotype VNII (21.2 %) and only one strain represented genotype VNIV (3 %). The high prevalence of genotype VNI is in accordance with other studies and this genotype is noted for having a worldwide distribution (Meyer *et al*., 1999).

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5. CONCLUSIONS

Our results indicate that *C. neoformans* var. *grubii* (serotype A), MATα, genotype VNI is the predominant strain associated with both clinical and environmental sources within South Africa. All of these results are in accordance with previous and current literature stating that *C. neoformans* var. *grubii* (serotype A, MATα, VNI) is responsible for approximately 90 % of all cryptococcal infections globally (Mitchell and Perfect, 1995) and 88 % of cryptococcal infections within the Gauteng area alone (Meyer *et al*., 1999).

In future extensive isolation and identification programs need to be initiated to further examine the distribution of *C. neoformans* within the South African environment in order to determine potential high risk exposure areas for concern of our increasing HIV and AIDS populations. Also, to comprehend the forces impacting on the distribution of this pathogen, future studies should focus on the uneven distribution of genotypes and mating types among clinical and environmental isolates of *C. neoformans* var. *grubii*. Finally, in order to understand the apparent infrequent natural occurrence of *C. neoformans* var. *grubii*, growth and survival studies should be conducted in media simulating the suspected ecological niche of this opportunistic fungal pathogen.

Table 2 Sequence identification and determination of serotype, mating type and genotype of *C. neoformans* strain originating within the Gauteng, North West and Western Cape provinces of South Africa.

Reference Number	ITS Sequencing Identification	Serotype	Mating Type	Genotype			
				RFLP-PLB1	Micro-satellite $(GACA)_4$	Mini-satellite (M13)	RAPDS
ABO[1]-C13	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
ABO-C14	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
ABO-CF	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
CBS[2] 10571	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
CBS 10572	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
CBS 10573	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
CBS 10574	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC[3] 8855	*C. neoformans* var. *grubii*	A	MATα	VNII	VNII	VNII	VNII
MRC 8856	*C. neoformans* var. *neoformans*	D	MATα	VNIV	VNIV	VNIV	VNIV
MRC 8857	*C. neoformans* var. *grubii*	A	MATα	VNII	VNII	VNII	VNII
MRC 8858	*C. neoformans* var. *grubii*	A	MATα	VNII	VNII	VNII	VNII
MRC 8859	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8860	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8861	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8862	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8863	*C. neoformans* var. *grubii*	A	MATα	VNII	VNII	VNII	VNII
MRC 8864	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8865	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8866	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI

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Table 2 (Continued) Sequence identification and determination of serotype, mating type and genotype of *C. neoformans* strain originating within the Gauteng, North West and Western Cape provinces of South Africa.

Reference Number	ITS Sequencing Identification	Serotype	Mating Type	Genotype			
				RFLP-PLB1	Micro-satellite $(GACA)_4$	Mini-satellite (M13)	RAPDS
MRC[3] 8867	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8868	*C. neoformans* var. *grubii*	A	MATα	VNII	VNII	VNII	VNII
MRC 8878	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8879	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8880	*C. neoformans* var. *grubii*	A	MATα	VNII	VNII	VNII	VNII
MRC 8882	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8883	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8884	*C. neoformans* var. *grubii*	A	MATα	VNII	VNII	VNII	VNII
MRC 8885	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8887	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8888	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8889	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8890	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI
MRC 8891	*C. neoformans* var. *grubii*	A	MATα	VNI	VNI	VNI	VNI

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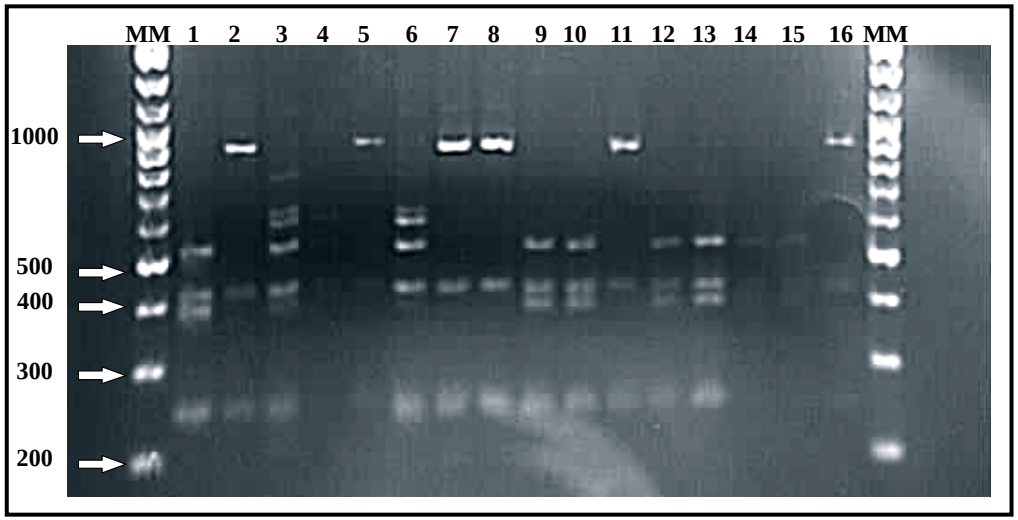

Figure 4 RFLP analysis of the Phospholipase B1 gene using the AvaI enzyme.

Lane 1 *C. neoformans* var. *grubii* CBS 10085 (VNI); Lane 2 *C. neoformans* var. *grubii* CBS 10084 (VNII); Lane 3 *C. neoformans* var. neoformans CBS 10079 (VNIV); Lane 4 *C. neoformans* CBS 10080 (VNIII); Lane 5 MRC 8855 (VNII), Lane 6 MRC 8856 (VNIV), Lane 7 MRC 8857 (VNII), Lane 8 MRC 8858 (VNII), Lane 9 MRC 8861 (VNI), Lane 10 MRC 8862 (VNI), Lane 11 MRC 8863 (VNII), Lane 12 MRC 8864 (VNI), Lane 13 MRC 8865 (VNI), Lane 14 MRC 8866 (VNI), Lane 15 MRC 8867 (VNI), Lane 16 MRC 8868 (VNII). MM MM Molecular marker, O'GeneRuler^{TM} DNA ladder mix (Fermentas, Cat no: #SM1173, 0.1 µg/µL).

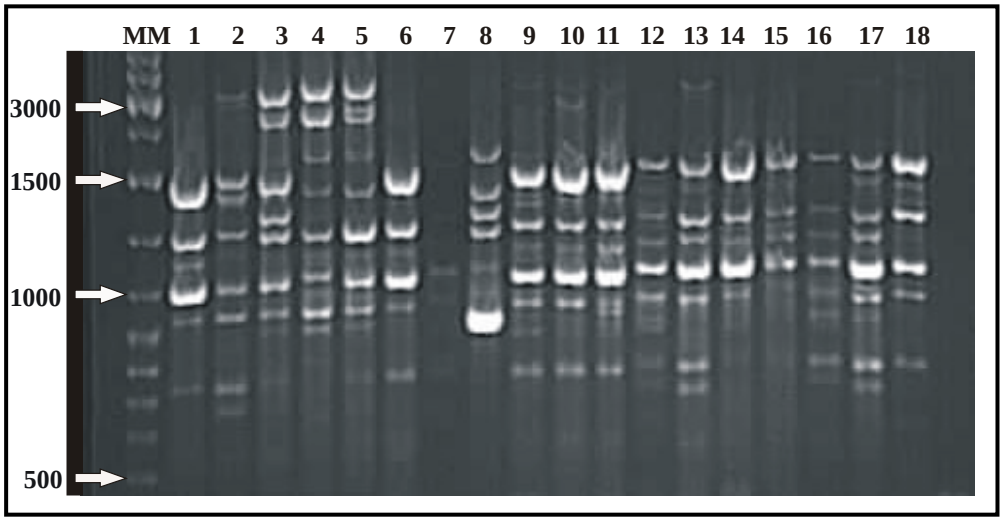

Figure 5 Micro-satellite PCR fingerprinting using the single primer $(GACA)_4$.

Lane 1 *C. neoformans* var. *grubii* CBS 10085 (VNI); Lane 2 *C. neoformans* var. *grubii* CBS 10084 (VNII); Lane 3 *C. neoformans* var. neoformans CBS 10079 (VNIV); Lane 4 *C. neoformans* CBS 10080 (VNIII); Lane 5 CBS 132 (VNIV); Lane 6 H99 (VNI); Lane 7 CBS 10751 (VNI); Lane 8 CBS 10572 (VNI); Lane 9 CBS 10574 (VNI); Lane 10 CBS 10573 (VNI); Lane 11 MRC 8878 (VNI); Lane 12 MRC 8879 (VNI); Lane 13 MRC 8880 (VNII); Lane 14 MRC 8882 (VNI); Lane 15 ABO-CF (VNI); Lane 16 MRC 8883 (VNI); Lane 17 MRC 8884 (VNII); Lane 18 MRC 8885 (VNI); MM Molecular marker, O'GeneRuler^{TM} DNA ladder mix (Fermentas, Cat no: #SM1173, 0.1 µg/µL).

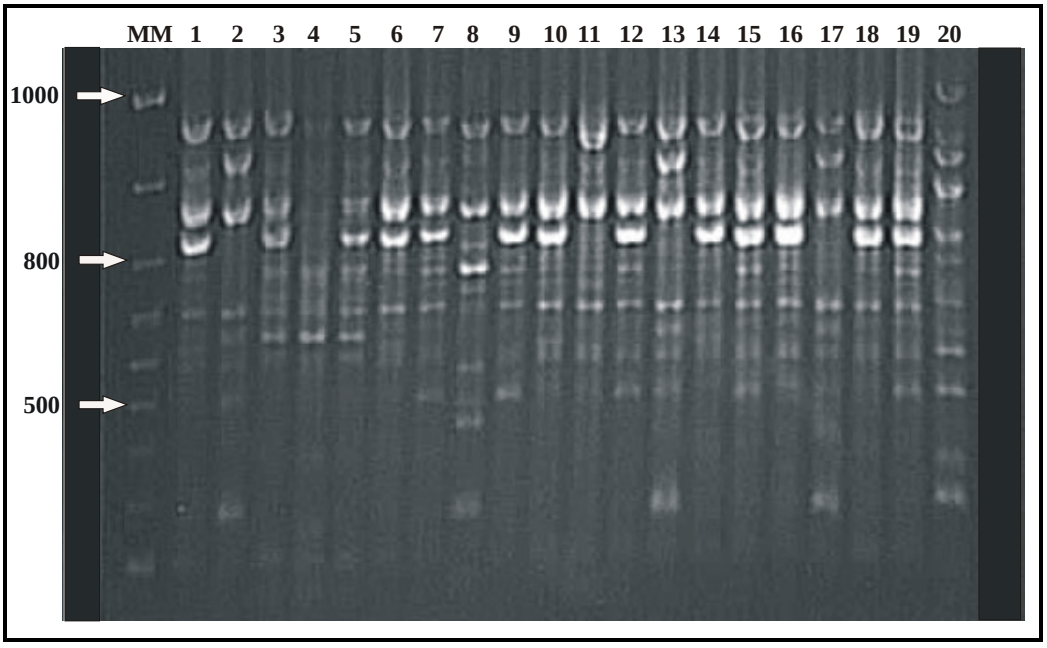

Figure 6 Mini-satellite PCR fingerprinting using the single primer M13.

Lane 1 *C. neoformans* var. *grubii* CBS 10085 (VNI); Lane 2 *C. neoformans* var. *grubii* CBS 10084 (VNII); Lane 3 *C. neoformans* var. neoformans CBS 10079 (VNIV); Lane 4 *C. neoformans* CBS 10080 (VNIII); Lane 5 CBS 132 (VNIV); Lane 6 H99 (VNI); Lane 7 CBS 10751 (VNI); Lane 8 CBS 10572 (VNI); Lane 9 CBS 10574 (VNI); Lane 10 CBS 10573 (VNI); Lane 11 MRC 8878 (VNI); Lane 12 MRC 8879 (VNI); Lane 13 MRC 8880 (VNII); Lane 14 MRC 8882 (VNI); Lane 15 ABO-CF (VNI); Lane 16 MRC 8883 (VNI); Lane 17 MRC 8884 (VNII); Lane 18 MRC 8885 (VNI); Lane 19 MRC 8887 (VNI); Lane 20 MRC 8888 (VNI); MM Molecular marker, O'GeneRuler^{TM} DNA ladder mix (Fermentas, Cat no: #SM117, 0.1 µg/µL).

6. LITERATURE CITED

Barreto de Oliveira M. T., Boekhout T. Theelen B., Hagen F., Baroni F. A., Lazéra M. S., Lengeler K. B., Heitman J., Rivera J. N. G., Paula C. R. (2004). *Cryptococcus neoformans* shows a remarkable genotypic diversity in Brazil. *J. Clin. Microbiol*. **42, 3:** 1356-1359.

Boekhout T, van Belkum A., Leenders A. C. A. P., Verbrugh H., Mukamurangwa P., Swinne D., Scheffers W. A. (1997). Molecular typing of *Cryptococcus neoformans*: taxonomic and epidemiological aspects. *Int. J. Syst. Bacteriol*. **47, 2:** 432-442.

Boekhout T., Theelen B., Diaz M., Fell J. W., Hop W. C. J., Abeln E. C. A., Dromer F., Meyer W. (2001). Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiol.* **147:** 891-907.

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready made' virulence and 'dual use' virulence factors in pathogenic environment-the *Cryptococcus neoformans* paradigm. *Curr. Opin. Microbiol.* **6**, 332-337.

Chaturvedi S., Rodeghier B., Fan J., McClelland C. M., Wickes B. L., Chaturvedi V. (2000). Direct PCR of *Cryptococcus neoformans* MAT*a* and MATα pheromones to determine mating type, ploidy, variety: a tool for epidemiological and molecular pathogenesis studies. *J. Clin. Microbiol*. **38:** 2007-2009.

Chen S. C., Currie B. J., Campbell H. M., Fisher D. A., Pfeiffer T. J., Ellis D. H., Sorrell T. C. (1997). *Cryptococcus neoformans* var. *gattii* infection in northern Australia: existence of an environmental source other than known host eucalypts. *Trans. R. Soc. Trop. Med. Hyg.* **91, 5:** 547-550.

Chory J., Pollard J. D. Jr. (1999). Resolution and recovery of small DNA fragments. In: Chory J., Pollard J. D. Jr. (Eds.) *Current Protocols in Molecular Biology.* **2:** 2.7.1- 2.7.8.

Chuck S. L., Sande M. A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **321:** 794–799.

Cogliati M., Esposto M. C., Clarke D. L., Wickes B. L., Viviani M. A. (2001). Origin of *Cryptococcus neoformans* var. *neoformans* diploid strains. *J. Clin. Microbiol*. **39, 11:** 3889-3894.

Dromer F., Mathoulin S., Dupont B., Letenneur L., Ronin O. (1996) Individual and environmental factors associated with infection due to *Cryptococcus neoformans* serotype D. French Cryptococcosis Study Group. *Clin. Infect. Disease.* **24, 4:** 744-745.

Ellis D. H., Pfeiffer T. J. (1990). Ecology, life cycle and infectious propagule of *Cryptococcus neoformans. Lancet.* **336:** 923–925.

Filion T., Kidd S., Aguirre K. (2006). Isolation of *Cryptococcus laurentii* from Canada goose guano in rural upstate New York. *Mycopathologia*. **162, 5:** 363-368.

Franzot S. P., Hamdan J. S., Currie B. P., Casadevall A. (1997). Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: Evidence for both local genetic differences and a global clonal population structure. *J. Clin. Microbiol.* **35, 9:** 2243-2251.

Franzot S. P., Fries B. C., Cleare W., Casadevall A. (1998). Genetic relationship between *Cryptococcus neoformans* var. *neoformans* strains of serotypes A and D. *J. Clin. Microbiol*. **36, 8:** 2200-2204.

Fraser J. A., Giles S. S., Wenink E. C., Geunnes-Boyer S. G., Wright J. R., Diezmann S., Allen A., Stajichl J. E., Dietrichl F. S., Perfect J. R., Heitman J. (2005). Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature Lett.* **437, 27:** 1360-1364.

Goldman D. L., Khine H., Abadi J., Lindenberg D. J., Pirofski L., Njang R., Casadevall A. (2001). Serologic evidence for *Cryptococcus neoformans* infection in early childhood. *Pediatrics.* **107:** 66-73.

Granados D. P., Castaneda E. (2006). Influence of climatic conditions on the isolation of members of the *Cryptococcus neoformans* species complex from trees in Colombia from 1992-2004. *FEMS Yeast Res.* **6:** 636-644.

Halliday C., Bui T., Krockenberger M., Malik R., Ellis D., Carter D. (1999). Presence of α and *a* mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gatti* strains from Australia. *J. Clin. Microbiol.* **37,** 2920-2926.

Hsu M. M., Chang J. C., Yokoyama K., Nishimura K., Miyaji M. (1994). Serotypes and mating types of clinical strains of *Cryptococcus neoformans* isolated in Taiwan. *Mycopathologia.* **125, 2:** 77-81.

Husain S., Wagener M. M., Singh N. (2001). *Cryptococcus neoformans* infection in organ transplant recipients: variables influencing clinical characteristics and outcome. *Emerg. Infect. Dis.* **7:** 375-381.

Ikeda R., Sugita T., Jacobson E. S., Shinoda T. (2002). Laccase and melanization in clinically important *Cryptococcus* species other than *Cryptococcus neoformans*. *J. Clin. Microbiol*. **40, 4:** 1214-1218.

Kligman A. M., F. D. Weidman. (1949). Experimental studies on treatment of human torulosis. *Arch. Derm. Syph*. **60:** 726–741.

Kohno S., Varma A., Kwon-Chung K. J., Hara K. (1994). Epidemiology studies of clinical isolates of *Cryptococcus neoformans* of Japan by restriction fragment length polymorphism. *Kansenshogaku Zasshi*. **68, 12:** 1512-1517.

Kwon-Chung K. J. (1975). A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. *Mycologia.* **61, 6:** 1197-1200.

Kwon-Chung K. J. (1976). A new species of *Filobasidiella*, the sexual state of *Cryptococcus neoformans* B and C serotypes. *Mycologia*. **68:** 943- 946.

Kwon-Chung K. J., Polacheck I., Bennett J. (1982). Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotype A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). *J. Clin. Microbiol.* **15:** 535-537.

Kwon-Chung, K. J. & Bennett, J. E. (1984). Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. *Am. J. Epidemiol.* **120:** 123-130.

Kwon-Chung K. J., Edman J. C., Wickes B. L. (1992). Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infect. Immun.* **60:** 602-605.

Latouche G. N., Huynh M., Sorrell T. C., Meyer W. (2003). PCR-Restriction fragment length polymorphism analysis of the phospholipase B (*PLB1*) gene for sub-typing of *Cryptococcus neoformans* isolates. *Appl. Environ. Microbiol*. **69:** 2080-2086.

Lazéra M. S., Pires F. D., Camillo-Coura L., Nishikawa M. M., Bezerra C. C., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *J. Med. Vet. Mycol.* **34, 2:** 127-31.

Lengeler K. B., Wang P., Cox G. M., Perfect J. R., Heitman J. (2000). Identification of the MAT*a* mating-type locus of *Cryptococcus neoformans* reveals a serotype A MAT*a* strain thought to have been extinct. *Proc. Natl. Acad. Sci.USA.* **97, 26:** 14455–14460.

Litvinseva A. P., Thakur R., Vilgalys R., Mitchell T. G. (2006). Multilocus sequence typing reveals three genetic subpopulations of *Cryptococcus neoformans* var. *grubii* (Serotype A), including a unique population in Botswana. *Genet.* **172:** 2223-2238.

Martinez L. R., Garcia-Rivera J., Casadevall A. (2001). *Cryptococcus neoformans* var. *neoformans* (Serotype D) strains are more susceptible to heat than *C. neoformans* var. *grubii* (Serotype A) strains. *J. Clin. Microbiol.* **39, 9:** 3365-3367.

McClelland C. M., Fu J., Woodlee G. L., Seymour T. S., Wickes B. L. (2002). Isolation and characterization of the *Cryptococcus neoformans MATa* pheromone gene. *Genet.* **160:** 935-947.

Meyer W., Mitchell T. G., Freedman E. Z., Vilgalys R. (1993). Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase

chain reaction to distinguish strains of *Cryptococcus neoformans*. *J. Clin. Microbiol.* **31, 9:** 2274-2280.

Meyer W., Marszewska K., Amimostofian M., Igreja R. P., Hardtke C., Methling K., Viviani M. A., Chindamporn A., Sukroongreung S., John M. A., Ellis D. H., Sorrell T. C. (1999). Molecular typing of global isolates of *Cryptococcus neoformans* var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA - a pilot study to standardize techniques on which to base a detailed epidemiological survey. *Electrophoresis.* **20:** 1790-1799.

Meyer W., Castaneda A., Jackson S., Huynh M., Castaneda E., the IberoAmerican Cryptococcal Study Group. (2003). Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerg. Infect. Dis.* **9, 2:** 189-195.

Mitchell T. G., Perfect J. R. (1995). Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. *Clin. Microbiol. Rev.* **8, 4:** 515-548.

Nielsen K., Cox G. M., Wang P., Toffaletti D. L., Perfect J. R., Heitman J. (2003). Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic *a* and α isolates. *Infect. Immun.* **71, 9:** 4831-4841.

Okoli I., Oyeka C. A., Kwon-Chung K. J., Theelen B., Robert V., Groenewald J. Z., McFadden D. C., Casadevall A., Boekhout T. (2007). *Cryptotrichosporon anacardii* gen. nov., sp. nov., a new trichosporonoid capsulate basidiomycetous yeast from Nigeria that is able to form melanin on Niger seed agar. *FEMS Yeast Res.* **7:** 339-350.

Padhye A. A., Chakrabarti A., Chander J., Kaufman L. (1993). *Cryptococcus neoformans* var. *gattii* in India. *J. Med. Vet. Mycol.* **31, 2:** 165-168.

Perfect J. R., Ketabchi N., Cox G. M., Ingram C. W., Beiser C. L. (1993). Karyotyping of *Cryptococcus neoformans* as an epidemiological tool. *J. Clin. Microbiol.* **31, 12:** 3305-3309.

Petter R., Kang B. S., Boekhout T., Davis B. J., Kwon-Chung K. J. (2001). A survey of heterobasidiomycetous yeasts for the presence of the genes homologous to virulence factors of *Filobasidiella neoformans*, *CNLAC1* and *CAP59*. *Microbiol.* **147:** 2029-2036.

Poonwan N., Mikami Y., Poosuwan S., Boon-Long J., Mekha N., Kusum M., Yazawa K., Tanaka R., Nishimura K., Konyama K. (1997). Serotyping of *Cryptococcus neoformans* strains isolated from clinical specimens in Thailand and their susceptibility to various antifungal agents. *European J. Epidemiol.* **13:** 335-340.

Rozenbaum R., **Gonçalves A. J**., **Wanke B**., **Caiuby M. J**., **Clemente H**., **Lazéra Mdos S**., **Monteiro P. C**., **Londero A. T. (1992).** *Cryptococcus neoformans* varieties as agents of cryptococcosis in Brazil. *Mycopathologia.* **119, 3:** 133-136.

Soil Classification Working Group (1991). Soil classification: A taxonomic system for South Africa. *Memoirs on the Agricultural Natural Resources of South Africa,* **No. 15:** Department of Agricultural Development, Pretoria.

Sorrell T. C., Chen S. C. A., Ruma P., Meyer W., Pfeiffer T. J., Ellis D. H., Brownlee A. G. (1996). Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. *J. Clin. Microbiol.* **34, 12:** 1253-1260.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans*. *Rev. Iberoam. Microbiol*. **14:** 42-43.

Sugita T., Takashima M., Ikedo R., Nakase T., Shinoda T. (2000). Intraspecies diversity of *Cryptococcus laurentii* as revealed by sequences of internal transcribed spacer regions and 28S rRNA gene and taxonomic position of *C. laurentii* clinical isolates. *J. Clin. Microbiol.* **38, 4:** 1468-1471.

Tscharke R. L., Lazéra M., Chang Y. C., Wickes B. L., Kwon-Chung K. J. (2003). Haploid fruiting in *Cryptococcus neoformans* is not mating type α-specific. *Fungal Genet. Biol.* **39:** 230-237.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil, *Med. Mycol.* **41, 5:** 383-390.

Vilchez R., Shapiro R., McCurry K., Kormos R., Abu-Elmagd K., Fung J., Kusne S. (2003). Longitudinal study of cryptococcosis in adult solid-organ transplant recipients.*Transpl. Int.* **16:** 336-340.

Villanueva E., Mendoza M., Torres E., de Albornoz M. B., Cavazza M. E., Urbina G. (1989). Serotyping of 27 *Cryptococcus neoformans* strains isolated in Venezuela. *Acta. Cient. Venez.* **40, 2:** 151-154.

Viviani M. A., Esposto M. C., Cogliati M., Montagna M. T., Wickes B. L. (2001). Isolation of a *Cryptococcus neoformans* serotype A MAT*a* strain from the Italian environment. *Med. Mycol.* **39, 5:** 383-386.

Varma A., Swinne D., Staib F., Bennett J. E., Kwon-Chung K. J. (1995). Diversity of DNA fingerprints in *Cryptococcus neoformans. J. Clin. Microbiol.* **33, 7:** 1807-1814.

Wickes B. L., Mayorga M. E., Edman U., Edman J. C. (1996). Dimorphism and haploid fruiting in *Cryptococcus neoformans*: Association with the α-mating type. *Microbiol.* **93:** 7327-7331.

White T. J., Bruns T., Lee S., Taylor J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes M. A., Gelfand D. H., Sninsky J. J., Whites T. J.(Eds.) *PCR Protocols,* Academic Press, San Diego, pp 482.

Yarrow D. (1998). Methods for isolation, maintenance and identification of yeasts. In: Kurtzman C. P., Fell J. W. (Eds.) *The Yeasts, a Taxonomic Study*, Elsevier Science BV, Amsterdam, pp 77-98.

Chapter 3

Ontogeny of *Cryptococcus neoformans* var. *grubii* within a woody environment

Aspects of the following chapter have been submitted for publication in scientific Journal of Microbial Ecology

Ontogeny of *C. neoformans* var. *grubii* within a woody environment

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1. ABSTRACT

A total of 36 *C. neoformans* strains originating from the provinces of Gauteng, North West and the Western Cape, South Africa, were screened for wood degrading enzymes. All strains tested positive for cellulase activity, 6 % of strains tested positive for laccase production at a temperature of 22 ºC, but no strains were able to degrade xylanase. Subsequently, three *C. neoformans* var. *grubii* strains originating from clinical and environmental samples, all representing the same genotype (VNI) and mating type (MATα), were evaluated for growth on *Acacia mearnsii* and *Eucalyptus camaldulensis* debris. While minimal differences were noted between strains, those cultured on *A. mearnsii* yielded significantly higher cell numbers. Finally, all strains were mated on *A. mearnsii* and *E. camaldulensis* debris, as well as V8 juice and yeast carbon base agar (YCB) to determine whether *C. neoformans* strains were capable of both dikaryotic and monokaryotic fruiting when cultured on woody debris. A total of 19 %, 6 %, 42 % and 72 % of the *C. neoformans* strains were able to mate when crossed on *A. mearnsii* and *E. camaldulensis* debris, V8 juice and YCB agar, respectively. Monokaryotic fruiting was observed in 3 %, 3 % and 3 % of strains when *C. neoformans* was cultured on *A. mearnsii*, *E. camaldulensis* debris and YCB, respectively. This may be the first observation of *C. neoformans* in a hyphal phase when cultured on medium comprised solely of woody debris.

2. INTRODUCTION

Cryptococcus neoformans and *Cryptococcus gattii* are basidiomycetous yeasts belonging to the order *Tremellales* (Boekhout *et al*., 1997), the jelly fungi, of which

these two yeast species are the only known pathogens. Interestingly, both these yeast species are known to form the *Tremella* haustorial branches, suggesting that *C. neoformans* and *C. gattii* are primarily fungal parasites or saprophytes. Although being closely related, the two species differ in a variety of areas; the most striking of which are with regards to their host preference and environmental distribution. While *C. gattii* is largely associated with immuno-competent individuals, *C. neoformans* is prevalent within the immuno-suppressed population, particularly those suffering from the human immuno virus (HIV) and acquired immuno-deficiency syndrome (AIDS).

Cryptococcus neoformans is known to have a world-wide distribution and has in turn been isolated from a number of environmental sources including soil, vegetative debris and particularly decaying wood and the hollows of trees (Trilles *et al*., 2003; Randhawa *et al*., 2001; Lazéra *et al*., 1996). It is clear however, that the variety *C. neoformans* var. *grubii*, one of two recognized varieties of the species *C. neoformans*, is predominant with regards to environmental and clinical sources with approximately 80 % of all clinical cases in North America being attributed to this serotype (Kwon-Chung and Bennett, 1984). As a result, *C. neoformans* var. *grubii* is regarded as being more virulent than its counterpart *C. neoformans* var. *neoformans*.

Occurring primarily as a haploid unicellular yeast cell (Boekhout *et al*., 1997), *C. neoformans* is capable of maintaining a hyphal phase producing basidia and the proposed infectious propagules, the basidiospores (Ellis and Pfeiffer, 1990; Kwon-Chung, 1975). *Cryptococcus neoformans* has evolved a number of ontogenic pathways that result in the formation of basidiospores. The first pathway is defined as dikaryotic fruiting or mating and occurs when yeast cells of opposite mating type, MAT*a* or MATα, fuse and form hyphae with fused clamp connections (Kwon-Chung, 1975). A second pathway, observed primarily in MATα cells under nitrogen limiting conditions, involves the development of hyphae with un-fused clamp connections from a single cell where no mating has occurred (Wickes *et al*., 1996). Termed monokaryotic or haploid fruiting, this form of reproduction was thought to explain the dominance of MATα within environmental isolations, because they are considered to be more virulent than MAT*a* cells. However, experimentation involving several animal models has shown no difference in virulence between mating types of *C. neoformans* var. *grubii* (serotype A) (Nielsen *et al*., 2003), thereby implicating that several other virulence factors may be involved.

To date a variety of “dual use” virulence factors are thought to play a role during pathogenesis, as well as the survival of *C. neoformans* in an environmental setting. The acquisition and maintenance of these virulence factors is believed to be a result of environmental interactions (Steenbergen *et al*., 2001). Virulence factors that have been well characterised thus far include the development of a polysaccharide capsule, the production of the pigment melanin, as well as the expression of a laccase enzyme. The production of a polysaccharide capsule by *C. neoformans* protects the cell from desiccation (Aksenov *et al*., 1973) and phagocytosis (Steenbergen *et al*., 2001). Interestingly, Rivera and co-workers (1998) were able to demonstrate that capsule thickness varied according to the location of the yeast cells within the human body. The thickest capsules were found within the lungs where the capsule is known to inhibit phagocytosis and contribute to macrophage cytotoxicity by capsule shedding (Tucker *et al*., 2002; Feldmesser *et al*., 2000), ultimately resulting in persistent cryptococcal infections

Melanin is a negatively charged pigment that is produced by the cryptococcal laccase enzyme by oxidising phenolic compounds (Steenbergen *et al*., 2003). Localized within the cell wall of *C. neoformans*, melanin is thought to maintain cell wall integrity and protect the yeast cell against a number of factors that include ultraviolet light (Wang *et al*., 1994), temperature fluctuations (Rosas *et al*., 1997) heavy metals (Garcia-Rivera *et al*., 2001), oxidants (Williamson, 1997), enzyme degradation (Rosas *et al*., 2001), microbial peptides and anti-fungals (Doering *et al*., 1999) as well as phagocytosis (Steenbergen *et al*., 2001).

The laccase enzymes are polyphenol oxidases that are capable of oxidizing phenolic compounds and aromatic amines (Min *et al*., 2001). Unlike the basidiomycetous white rot fungi that secret laccase, the cryptococcal laccase is covalently bound to the outer membrane of the yeast cell (Zhu *et al*., 2001). This localization may help to facilitate the survival of *C. neoformans* within a woody environment. Lignin is a large polymer, unable of traversing the cell membrane. The localization of laccase would therefore allow direct contact between the yeast cell and the lignin, thus facilitating degradation. The laccase enzyme, coupled to the recent discovery of a cryptococcal cellulase (Loftus *et al*., 2005), further links *C. neoformans* to a potentially woody ecological niche.

In 2001, Steenbergen and co-workers were able to demonstrate that

C. neoformans cells were ingested by the soil amoebae *Acanthamoeba castellanii* resulting in death of the amoebae. Similarly in 2002, Mylonakis and co-workers were able to show that the nematode *Caenorhabditis elegans* was able to feed on a variety of non-pathogenic cryptococcal soil yeasts; however the ingestion of *C. neoformans* resulted in the nematodes' death. In both studies, the interactions between *C. neoformans* and its predator closely resembled those observed between the yeast and human macrophages (Casadevall *et al*., 2003; Feldmesser *et al*., 2001; Steenbergen *et al*., 2001).

From the above it is obvious that although *C. neoformans* is able to interact with a number of different organisms, its phytotypic and genotypic characteristics point to an ecological niche rich in lignocellulosic material which may periodically be subjected to desiccation and/or sunlight. Indeed this species has been isolated from inside hollows of tree trunks and it has been suggested the decaying wood within this habitat may act as substrate for the yeast (Trilles *et al*., 2003; Randhawa *et al*., 2000; Lazéra *et al*., 1996). However, the ability of the yeast to grow and survive, and produce its ontogenic stages on woody debris has never been evaluated.

With the above as background the objectives of this study were to firstly test whether clinical and environmental isolates of *C. neoformans* var. *grubii* are capable of growth and interaction with selected microbes when cultured on woody debris. Secondly to test the hypothesis that *C. neoformans* is capable of producing all its ontogenic stages on similar woody debris.

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3. MATERIALS AND METHODS

3.1. Strains and culture maintenance

Cryptococcus neoformans var. *neoformans* CBS 132, CBS 10511 (JEC20), CBS 10513 (JEC21) and *C. neoformans* var. *grubii* CBS10515 (H99), CBS 9172 were obtained from the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. The environmental *C. neoformans* var. *grubii* isolates obtained during this study, CBS 10571, CBS 10572, CBS 10573, CBS 10574, have been deposited into the culture collection of the CBS, Utrecht, The Netherlands. Clinical *C. neoformans* var. *grubii* and

C. neoformans var. *neoformans* were obtained from the culture collection of the Medical Research Council (MRC), PROMEC Unit, Tygerberg, South Africa (see Appendix A). A total of three *C. neoformans* var. *grubii* strains were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa (see Appendix A). The reference strains *Aspergillus niger* ABO-AN1, *Cryptococcus laurentii* ABO-1A, *Cryptococcus podzolicus* ABO-5A, *Pycnoporus* sp. ABO-P1, *Saccharomyces cerevisiae* ABO-SC1 and *Trichoderma reesei* ABO-TR1 were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa. All strains were maintained by periodic transfer to yeast peptone glucose (YPG, pH 5.0) agar (Yarrow, 1998) supplemented with 200 mg/L chloramphenicol (Sigma, Cat no: CO378-25G) and incubated at room temperature.

3.2. Screening for wood degrading enzymes

3.2.1. Laccase activity

All strains were inoculated onto yeast nitrogen base (with amino acids, Difco, Cat no: 239210) supplemented with 5 g/L D-glucose (Biolab) and 300 mg/L 2,2′-azino-*bis*-3-ethylbenz-thiazoline-6-sulfonic acid (ABTS, Sigma, Cat no: A1888-5G) (Butler *et al*., 2003; Min *et al*., 2001) as well as onto malt extract agar (MEA, Biolab) supplemented with 0.1 mL/L Guaiacol (Sigma, Cat no: G-5502) (Kiiskinen *et al*., 2004). Both ABTS and Guaiacol containing plates were incubated at 22 and 37 °C for one week and examined for the production of green or brown pigments, respectively. Plates inoculated with *Pycnoporus* sp. ABO-P1 served as positive control, while plates inoculated with *S. cerevisiae* ABO-SC1 served as negative control.

3.2.2. Cellulase activity

All strains were inoculated onto carboxymethylcellulose-agar (CMC, Sigma, Cat no: C-5678) plates and incubated at 22 °C for one week (De Koker *et al*., 2000). Plates were stained using 0.1 % congo red solution (B&M Scientific, Cat no: 107076), for 15 minutes and de-stained with a 1 M sodium chloride (NaCl, Biolab) solution for 15 minutes. Plates were examined for the production of clear zones indicative of enzyme activity. Plates inoculated with *T. reesei* ABO-TR1 served as positive control, while plates inoculated with *S. cerevisiae* ABO-SC1 served as negative control. All plates were inoculated in triplicate.

3.2.3. Xylanase activity

All strains were inoculated on Remazol Brilliant Blue -Xylan (Sigma, Cat no: R-8001) covalently linked to Beechwood 4-O-methyl–D-glucurono-D-xylan (Sigma, Cat no: X-0502) and incubated at 22 °C for one week (Farkas *et al*., 1985). Plates were examined for the production of clear zones around yeast colonies indicative of xylanase activity. Plates inoculated with *A. niger* ABO-AP1 served as positive control, while plates inoculated with *S. cerevisiae* ABO-SC1 as negative control. All plates were inoculated in triplicate.

3.3. Testing for growth of *C. neoformans* var. *grubii* on woody debris

3.3.1. Preparation of woody debris

Acacia mearnsii and *Eucalyptus camaldulensis* trees from the Stellenbosch area in the Western Cape, South Africa, were felled. Each tree was split into four components, namely the main stem, branches, twigs and leaves. The main stem was chipped with a Wigger pilot-size chipper, and a randomized chip sample was taken. The sampled stem material consisting of relative large wood chips, as well as the branch, twig and leave components, were dried for 24 hours at 100 ºC and put through a Condux-Hype type hammer mill giving smaller chips, 10 x 1 x 1 mm in dimension. The smaller-sized chips of all four tree components were sub-sampled and each sample was further reduced in size, with a Retsch (ZM-1) ultra centrifugal mill with a 6 mm sieve, resulting in roughly the same particle size distribution for each of the four components. The four components were subsequently mixed in a ratio of 50 % (w/w) main stem, 16 % (w/w) branches, 16 % (w/w) twigs and 16 % (w/w) leaves.

Red clay, donated by Corobrik Brick Works (Pty) Ltd, Stellenbosch, South Africa was dried in an oven at 100 ºC for 48 hours, ground using a pestle and mortar and sieved using a 2.0 mm sieve.

Silica sand was washed using a 1 M hydrochloric acid solution, rinsed twice using distilled water and dried at 100 ºC for 48 hours.

3.3.2. Physico-chemical analysis of woody substrate

To determine the field capacity, a total of six vertical columns (10 mm in diameter and 30 mm in length) were set up containing various combinations of woody substrate. Columns 1 and 2 contained *E. camaldulensis* and *A. mearnsii* debris,

respectively. Columns 3 and 4 contained a combination of *E. camaldulensis* debris and clay, and *A. mearnsii* debris and clay, respectively. Finally columns 5 and 6 contained *E. camaldulensis* debris and silica sand, and *A. mearnsii* debris and silica sand, respectively. Where two components were combined, a ratio of 1:1 (w/w) was applied to produce a woody substrate. Each column was saturated with approximately 300 mL water and allowed to dry by means of gravitation for 48 hours. The substrate was removed from each column, weighed and dried at 110 °C for 24 hours after which the dried substrate was subsequently weighed for a second time. The field capacity was calculated as a percentage (w/v) with regards to the amount of water absorbed and then lost by the substrate (British Columbia, 2002; Abbott, 1985).

The organic carbon of each woody substrate was determined using the Walkey-Black method (Nelson and Sommers, 1982). The total nitrogen content of each woody substrate was determined by digesting it in a LECO FP-528 nitrogen analyser, while the ammonium and nitrate content was determined in a 1 M KCl extract (Bremner, 1965). Bray-2 extract (Thomas and Peaslee, 1973) was used to determine the phosphate, while di-ammonium EDTA extract (Beyers and Coetzer, 1971) was used to determine copper, zinc and manganese content of each woody substrate. Boron was determined in a hot water extract (Fertilizer Society of South Africa, 1974). The exchangeable cations (calcium, magnesium, potassium and sodium) were determined using a 1 M ammonium acetate extract and the cation exchange capacity of each woody substrate was calculated (Doll and Lucas, 1973).

3.3.3. Preparation of woody debris solid state cultures containing C. neoformans *var*. grubii

Pre-inoculums were prepared from each of the strains of the *C. neoformans* var. *grubii*, namely H99, CBS 10571 and MRC 8890. The strains were cultured at 26 °C for 48 hours in 250 mL conical flasks, containing 25 mL yeast malt broth (YM, pH 5.0) on a rotary shaker (setting of 4). A total of 2 mL of each culture was centrifuged (Biofuge fresco, Heraeus Instruments) for 5 min at 13 793 RCF, and the pellet was re-suspended in 2 mL sterile distilled water. Total cell counts of the resulting suspensions that served as final inoculums were done using a Neubauer haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen).

A series of woody substrates were prepared in 50 mL screw capped glass jars. The jars, each containing 10 g substrate, were autoclaved (121 °C, 15 min) and dried

in an oven at 50 °C for 48 hours. Where two components were combined to produce the woody substrate, a ratio of 1:1 (w/w) was applied.

Each series of woody substrates was subsequently inoculated with a single *C. neoformans* var. *grubii* strain, namely H99, CBS 10571 or MRC 8890, to a final concentration of 7.5 x 10^5 yeast cells per gram of woody substrate. The field capacity of each microcosm was adjusted using sterile distilled water; jars were vortexed and incubated at 26 °C in a sealed 100 x 600 x 300 mm plastic Tupperware container. Sterile distilled water was added to the plastic Tupperware container to ensure high moisture content.

After an hour, a total of 1 g solid state culture was sampled from each jar and yeasts were enumerated using dilution plates with malt extract agar (MEA, Biolab) supplemented with 200 mg/L chloramphenicol (Sigma, Cat no: CO378-25G). Plates were incubated at 30 °C for 48 hours before the colonies were counted. Enumeration of yeasts was repeated on days 4, 7, 10, 14, 20 and 30. Data obtained were plotted on a log graph using Microsoft Office Excel 2003.

3.4. Survival of *C. neoformans* var. *grubii* in woody substrate in the presence of selected microbes

The survival of *C. neoformans* var. *grubii* was determined in the presence of selected microbes that may occur naturally in the same ecological niche of this pathogen. Both bacterial species and predatory protists were included in the experimentation.

3.4.1. Enrichment for Protista

Using enrichment procedures, protists were isolated from soil or rotting wood. A pre-inoculum containing a consortium of environmental microbes, was prepared by adding 10 g of soil or rotting wood to 90 mL distilled water, shaking vigorously for 10 min and then allowing the suspension to settle for 96 hours at 22 °C. A total of 4 mL supernatant was subsequently transferred to 20 mL sterile soil solution supplemented with 0.6 mg/L penicillin (Sigma, Cat no: PEN-K). Finally, 1 mL of the selected yeast culture was added as “bait” for protista. The resulting enrichment cultures were incubated at 22 °C in the dark.

After one week of incubation, 4 mL of enrichment culture was transferred via sterile glass wool to fresh sterile soil solution supplemented with 0.5 mg/L

streptomycin (Sigma, Cat no: S6501-50G). This process was repeated monthly and growth of the protistan populations within the cultures was monitored microscopically.

3.4.2. Preparation of Sterile Soil Solution and C. neoformans *var*. grubii *cultures*

Sterile soil solution used during the enrichment for protista, was prepared by mixing 100 g soil in 900 mL distilled water, shaking vigorously for 10 minutes and allowing the suspension to settle for 48 hours at room temperature. The resulting supernatant was filtered through Whatman 2 filter paper (Whatman, Cat no: 1002150) and sterilized using an autoclave (121 °C; 15 min).

C. neoformans var. *grubii* cells used as "bait" during the enrichment for protista were obtained by inoculating strains representing *C. neoformans* var. *grubii*, namely H99, CBS 10571, MRC 8890, into 25 mL YM broth (pH 5.0) contained in 250 mL conical flasks. The resulting cultures were incubated at 26 °C for 48 hours on a rotary shaker (setting of 4). The cultures were centrifuged (Biofuge Fresco, Heraeus Instruments) for 5 min (13 793 RCF), and the resulting pellets were washed three times by consecutive re-suspension in sterile distilled water and centrifugation (5 min at 13 793 RCF).

3.4.3. Preparation of woody substrate solid state cultures containing C. neoformans *var.* grubii *and protista*

A series of woody substrates comprising either *E. camaldulensis* and silica sand or *A. mearnsii* and silica sand were prepared as previously described.

Each series of woody substrates were subsequently inoculated with a single *C. neoformans* var. *grubii* strain, namely H99, CBS 10571 or MRC 8890 (obtained from pre-inoculum cultures as previously described), to a final concentration of 7.5×10^5 yeast cells per gram of woody substrate. Approximately 500 µL of predatory protists were added to each series of woody substrate from enrichment cultures. The field capacity of each microcosm was adjusted using sterile distilled water; jars were vortexed and incubated at 26 °C in a sealed 100 x 600 x 300 mm plastic Tupperware container. Sterile distilled water was added to the plastic Tupperware container to ensure high moisture content.

The enumeration of yeast was achieved as previously described. Enumeration of yeasts was repeated on days 4, 7, 10 and 14.

Protista were enumerated at the same time as yeasts by means of the most probable number (MPN) technique. Six-fold dilutions in microtiter plates containing sterile soil solution as medium (Ekelund *et al*., 1998) and examined for the presence of protists using an inverted microscope. Microtiter plates were subsequently incubated at 22 °C in the dark for a further 4 days, and then re-examined for the presence of protista. The MPN was calculated using the method described by Briones and co-workers (1999). Data obtained during the enumeration of the yeasts and protozoa were plotted on a log graph using Microsoft Office Excel 2003.

3.4.4. Isolation and identification of bacteria

Enrichment cultures for bacteria were prepared in a similar manner to that explained above; however, the addition of yeast cells as 'bait' was omitted. Rather, these cultures were supplemented with 10 g/L of either *A. mearnsii* or *E. camaldulensis* debris. After an incubation period at 26 °C for 48 hours; a total of 1 mL was sampled from each enrichment culture, a dilution serious prepared using saline solution and plated onto tryptic soy agar (TSA, Biolab) plates. Plates were incubated at 30 °C for 48 hours. Randomly selected bacterial colonies were streaked onto TSA plates and incubated at 30 °C for 48 hours. Morphological representatives from each enriched culture were randomly selected for further identification and characterization. These bacterial isolates were purified by consecutive streaking out of single colonies and cultivation on TSA plates incubated at 30 °C.

3.4.4.1. Genomic DNA isolation. Genomic DNA was isolated by the CTAB/NaCl mini-prep protocol (Ausubel *et al*., 1989).

3.4.4.2. Analysis of the 16S ribosomal DNA (rDNA) region. Amplification of the 16S rDNA region was done using universal 16S rDNA primers as previously described by Heyndrickx and co-workers (1996) and was achieved using a GeneAmp PCR System (Applied Biosystems, model 2400). Amplified products were separated in an agarose gel (0.8 %, w/v) and visualized by ethidium bromide staining according to standard methods (Chory and Pollard, 1999).

PCR products were purified with Nucleospin® Extract II (Macherey-Nagel, Cat no: 740 609.50) chromatography columns. Sequences representing the 16S

region of the rDNA from the strains were obtained using an ABI PRISM model 3100 genetic sequencer. The forward and reverse sequences were aligned with DNAMAN for WINDOWS Version 4.13 (Lynnon Biosoft). The bacterial strains were identified by comparing the sequencing results with known sequences using the BLAST program (www.ncbi.nlm.nih.gov/blast).

3.4.5. Preparation of woody substrate solid state cultures containing Pseudomonas fluorescens *or* Enterobacter sp.

Pre-inoculums were prepared from each of the bacterial strains, namely *P. fluorescens* and *Enterobacter* sp. The bacterial strains were inoculated into 25 mL tryptic soy broth (TSB, Biolab) contained in 250 mL conical flasks and incubated at 26 °C on a shaker for 24 hours. A total of 2 mL of each culture was centrifuged (Biofuge fresco, Heraeus Instruments) for 5 min at 13 793 RCF and the pellet was re-suspended in sterile distilled water. The resulting suspensions that served as the final inoculums were diluted with sterile distilled water in order to achieve an absorbance value of 1 at a wavelength of 550 nm though a standard 1 cm cuvette (Ultrospec 3000 UV/Visible Spectrophotometer; Pharmacia Biotech).

A series of woody substrates comprising either *E. camaldulensis* and silica sand or *A. mearnsii* and silica sand were prepared as previously described. Approximately 50 µL of *P. fluorescens* and *Enterobacter* sp. suspension was added to each series of *A. mearnsii* and *E. camaldulensis* woody substrates respectively. The field capacity of each woody substrate was adjusted using sterile distilled water; jars were vortexed and incubated at 26 °C in a sealed 100 x 600 x 300 mm plastic Tupperware container. Sterile distilled water was added to the plastic Tupperware container to ensure high moisture content.

After an hour, a total of 1 g solid state culture was sampled from each jar and the bacteria were enumerated using dilution plates with TSA (Biolab). Plates were incubated at 30 °C for 24 hours before the colonies were counted. Enumeration of bacteria was repeated on days 4, 7, 10 and 14. Data obtained were plotted on a log graph using Microsoft Office Excel 2003.

3.4.6. Preparation of woody substrate solid state cultures containing C. neoformans *var.* grubii *and* Pseudomonas fluorescens *or* Enterobacter sp.

A series of woody substrates comprising either *E. camaldulensis* and silica sand or *A. mearnsii* and silica sand were prepared as previously described.

Each series of woody substrates were subsequently inoculated with a single *C. neoformans* var. *grubii* strain, namely H99, CBS 10571 or MRC 8890 (obtained from pre-inoculum cultures as previously described), to a final concentration of 7.5×10^5 yeast cells per gram of woody substrate. Approximately 50 µL of *P. fluorescens* and *Enterobacter* sp. suspension (obtained from pre-inoculum cultures as previously described) was added to each series of *A. mearnsii* and *E. camaldulensis* woody substrates respectively. The field capacity of each woody substrate was adjusted using sterile distilled water; jars were vortexed and incubated at 26 °C in a sealed 100 x 600 x 300 mm plastic Tupperware container. Sterile distilled water was added to the plastic Tupperware container to ensure high moisture content.

The enumeration of yeast and bacteria was achieved as previously described. Enumeration of yeast and bacteria was repeated on days 4, 7, 10 and 14. Data obtained during the enumeration of yeast and bacteria were plotted on a log graph using Microsoft Office Excel 2003.

3.4.7. Preparation of woody substrate solid state microcosms containing C. neoformans *var.* grubii, P. fluorescens, Enterobacter *sp. as well as predatory protists*

A series of woody substrates comprising either *E. camaldulensis* and silica sand or *A. mearnsii* and silica sand were prepared as previously described.

Each series of woody substrates were subsequently inoculated with a single *C. neoformans* var. *grubii* strain, namely H99, CBS 10571 or MRC 8890 (obtained from pre-inoculum cultures as previously described), to a final concentration of 7.5×10^5 yeast cells per gram of woody substrate. Approximately 500 µL of predatory protista (obtained from pre-inoculum cultures as previously described) was added to each series of woody substrate from enrichment cultures and 50 µL of *P. fluorescens* and *Enterobacter* sp. suspension (obtained from pre-inoculum cultures as previously described) was added to each series of *A. mearnsii* and *E. camaldulensis* woody substrates respectively. The field capacity of each woody substrate was adjusted using sterile distilled water; jars were vortexed and incubated at 26 °C in a sealed 100 x 600

x 300 mm Tupperware container. Sterile distilled water was added to the plastic Tupperware container to ensure high moisture content.

The enumeration of yeasts, protists and bacteria was achieved as previously described. Enumeration of yeasts, protists and bacteria was repeated on days 4, 7, 10 and 14. Data obtained during the enumeration of yeast, protists and bacteria were plotted on a log graph using Microsoft Office Excel 2003.

3.5. Fruiting of *C. neoformans* on woody debris

Acacia mearnsii and *Eucalyptus camaldulensis* debris (200 g/L) was suspended in 2 % water agar (w/v) (Biolab) and autoclaved (121 °C; 15 min). Plates were poured using Petri dishes (90 mm diameter) and allowed to solidify. All strains were inoculated onto four separate *A. mearnsii* and *E. camaldulensis* agar plates. Three plates representing each wood species were combined with one of three *C. neoformans* reference strains namely, JEC20 (*a*/D); JEC21 (α/D) and CBS 9172 (*a*/A); the fourth plate remained as a single inoculum. Plates were incubated at 25 °C for 3-5 weeks and observed weekly macroscopically and microscopically for the development of hyphae, clamp connections and basidiospores (Cogliati *et al*., 2001; Yarrow, 1998).

3.6. Fruiting of *C. neoformans* on standard media

All strains were tested for dikaryotic and monokaryotic fruiting on V8 juice (Cogliati *et al*., 2001; Yarrow, 1998) and yeast carbon base (YCB) agar (Yarrow, 1998; Wickes *et al*., 1996).

3.6.1. Fruiting of C. neoformans *on V8 juice agar*

All strains were inoculated onto four separate V8 juice agar plates (Yarrow, 1998). Matings were performed as previously described (Cogliati *et al*., 2001; Yarrow, 1998).

3.6.2. Fruiting of C. neoformans *on nitrogen limited media – Yeast Carbon Base*

All isolates were inoculated onto four separate Yeast Carbon Base (YCB, Difco, Cat no: 0391-15) agar plates (Yarrow, 1998; Wickes *et al*., 1996). Matings were performed as previously described (Cogliati *et al*., 2001; Yarrow, 1998).

4. RESULTS AND DISCUSSION

4.1. Screening for wood degrading enzymes

4.1.1. Laccase activity

Despite the fact that *C. neoformans* is well known to be a laccase producing yeast only two strains, namely *C. neoformans* var. *grubii* MRC 8861 (Fig 1a) and *C. neoformans* var. *grubii* MRC 8863 (Fig 1b), tested positive for the production of laccase when incubated at 22 °C. Interestingly, unlike the positive control, *Pycnoporus* sp. ABO-P1 (Fig 1c), the *C. neoformans* var. *grubii* colony itself denoted the positive colour reaction, not the surrounding media. This corresponds to the localization of the cryptococcal laccase that is known to be a cell wall bound enzyme (Zhu *et al*., 2001). However, the localization of the laccase enzyme may limit the exposure of the enzyme to the substrate and may be a possible indication as to why only a limited number of *C. neoformans* strains displayed a positive result. When *C. neoformans* strains were cultured on the same media and incubated at 37 °C, where laccase acts as a potent virulence factor, no positive results were observed. Interestingly, studies have suggested that laccase may not be required for survival of *C. neoformans* within extra-pulmonary sites of the human body, including the central nervous system (Noverr *et al*., 2004). Barluzzi and co-workers (2000) were able to demonstrate that *C. neoformans* laccase deficient cells were capable of growth within the central nervous system indicating the influence of other virulence factors. However, the enzyme has been strongly implicated in the yeasts' survival and replication within alveolar macrophages that ultimately leads to the pathogen's dissemination (Noverr *et al*., 2004).

4.1.2. Cellulase activity

All *C. neoformans* strains tested positive when cultured on CMC agar; however, a clear zone was only visible directly below the yeast colony (Fig 2). In 2005, Loftus and co-workers were able to detect both a cellulase (CNE03150) and exo-beta-1,3-glucanase (CNL04840) gene within the genome of *C. neoformans*. Interestingly, a number of studies have focused on the extra-cellular activity of *C. neoformans* and none reported any cellulase activity (Vidotto *et al*., 2000). These findings, coupled to the location of the clear zone produced by all *C. neoformans* strains on CMC agar during the present study, suggest that similar to the laccase

enzyme, these cellulolytic enzymes may be cell wall bound. Alternatively, the enzymes may become trapped within the thick polysaccharide capsule produced by *C. neoformans* limiting contact with the surrounding cellulose.

The presence of these cellulose degrading enzymes coupled with the lignin degrading laccase enzyme, would serve as a huge advantage to *C. neoformans* within a woody environment. The close proximity of the enzyme action would also limit the scavenging of degraded cellulose by other micro-organisms in what is already regarded as a nutrient-limited habitat. A similar phenomenon occurs among biofilm-associated micro-organisms, where it is known that the localization of exo-enzymes, within exo-polymer capsules enhances the competitiveness of these micro-organisms in oligotrophic environments (Decho, 1990).

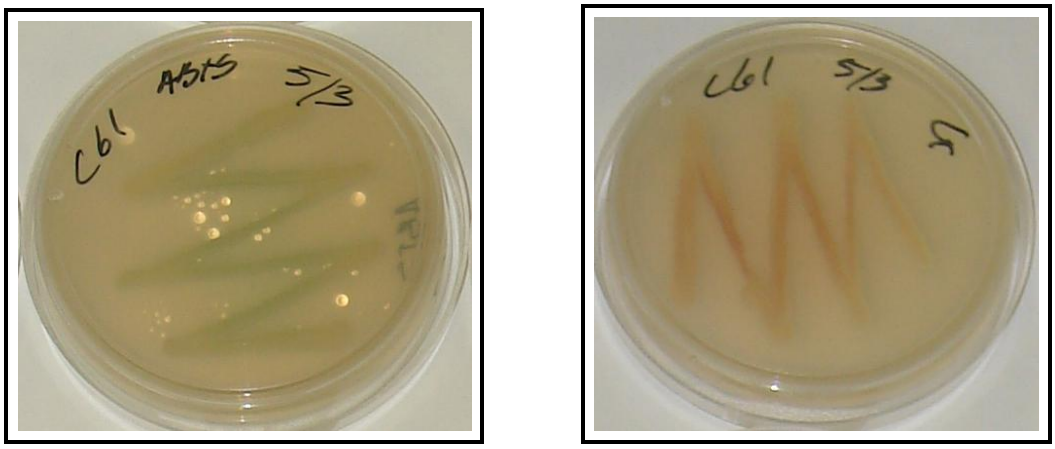

Figure 1a *C. neoformans* var. *grubii* MRC 8861 displaying positive green and brown pigmentation of the colony when cultured on ABTS and Guaiacol agar, respectively.

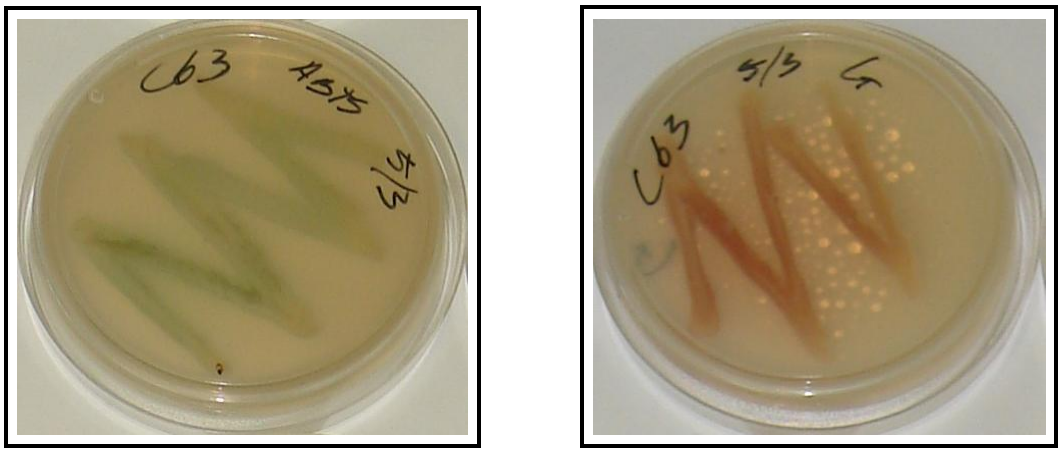

Figure 1b *C. neoformans* var. *grubii* MRC 8863 displaying positive green and brown pigmentation of the colony when cultured on ABTS and Guaiacol agar, respectively.

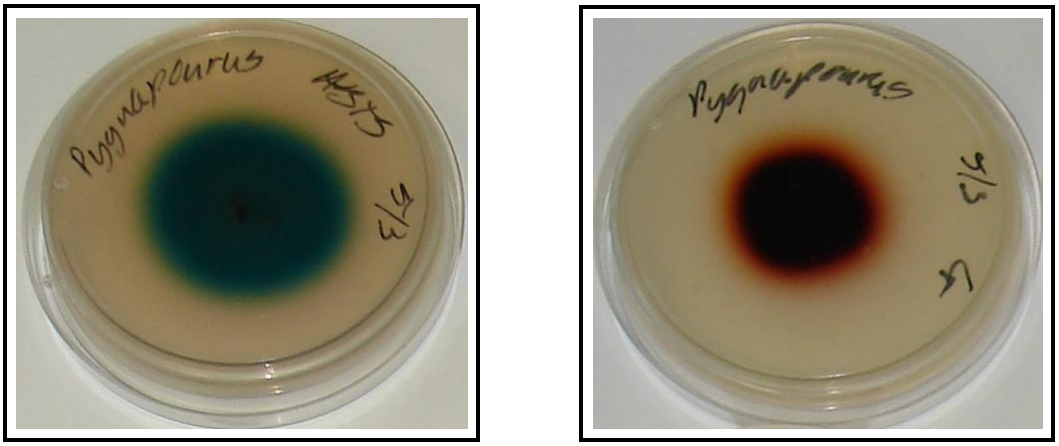

Figure 1c Positive control *Pycnoporus* sp. displaying positive green and brown pigmentation of the media when cultured on ABTS and Guaiacol agar, respectively.

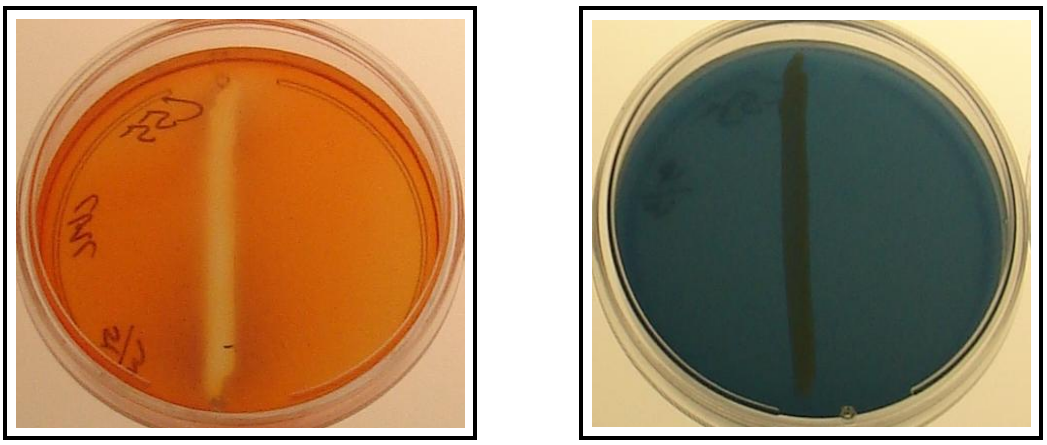

Figure 2 Clear zone produced by *C. neoformans* var. *grubii* MRC 8855 when cultured on CMC agar. No clear zone is produced when *C. neoformans* var. *grubii* MRC 8855 is cultured on RBB-Xylan agar.

4.1.3. Xylanase activity

All strains tested negative when cultured on RBB-xylan blue agar plates (Fig 2). Xylan, a linear polysaccharide chain comprised of xylose residues linked by β-1,4-bonds in the backbone, forms the major component of hemicellulose. Although *C. neoformans* is capable of xylose assimilation (Kwon-Chung, 1998); the yeast appears to be incapable of degrading xylan.

4.2. Testing for the growth of *C. neoformans* var. *grubii* on woody substrate

4.2.1. Physico-chemical analysis of woody substrate

Field capacity of soil is defined as the water content when all free water has been drained through gravity (British Columbia, 2002). However, values will differ depending on the soil texture and structure (Prichard, 2005). Sandy soils will drain relatively rapidly as opposed to clay soils that are easily water-logged. The field capacity was determined for each combination of woody substrate (Table 1). Woody substrates comprising solely of woody debris displayed the highest field capacity, while the combination containing red clay displayed a higher field capacity than those containing silica sand, possibly due to the adsorptive nature of the clay.

Chemical analysis revealed that *A. mearnsii* debris displayed significantly lower levels of the trace elements magnesium, manganese and zinc than *E. camaldulensis* (Table 2), however it contained more than twice the amount of nitrogen than the latter debris. This can be attributed to the fact that *A. mearnsii* is known to be a nitrogen fixing plant (Forrester *et al*., 2007) that is often incorporated into plantations in order to increase the levels of bio-available nitrogen. From Table 2 it is also obvious that the addition of either clay or sand to the woody debris resulted in the dilution of the nutrients.

Table 1 Field capacity determined for six combinations of woody substrate.

Debris 1:1 (w/w)	**Weight before drying (g)**	**Weight after drying (g)**	**Field Capacity (%)**
Eucalyptus debris	71.18	24.74	65.2
Eucalyptus debris and red clay	104.31	50.97	51.1
Eucalyptus debris and silica sand	102.92	61.52	40.2
Acacia debris	66.08	25.06	62.1
Acacia debris and red clay	107.03	52.02	51.4
Acacia debris and silica sand	90.52	53.07	41.4

Table 2 Chemical properties determined for six combination of woody debris. Values represent the mean of three samples while values in brackets represent standard deviations.

Substrate	**Avg[1] pH (KCl)**	**Avg P Bray II (mg/kg)**	**Avg K (mg/kg)**	**Avg Exchangeable cations (cmol(+)/kg)**				**Avg Cu (mg/kg)**	**Avg Zn (mg/kg)**	**Avg Mn (mg/kg)**	**Avg B (mg/kg)**	**Avg N (%)**	Avg C (%)
				Na	**K**	**Ca**	**Mg**						
Eucalyptus debris	4.3 (0.0)	160 (10.8)	3351.3 (113.1)	3.2 (0.2)	8.6 (0.3)	5.4 (0.6)	12.3 (0.9)	1.3 (0.3)	8.5 (0.3)	305.8 (10.5)	7.4 (1.3)	0.4 (0.04)	53.0 (0.2)
Eucalyptus debris and red clay	4.3 (0.0)	90.3 (11.2)	1988.3 (40.5)	2.1 (0.04)	5.1 (0.1)	4.3 (0.2)	8.2 (0.1)	1.9 (0.6)	6.1 (0.4)	226.6 (14.6)	3.8 (0.5)	0.2 (0.02)	27.0 (3.8)
Eucalyptus debris and silica sand	4.4 (0.06)	92.7 (11)	1768 (157.4)	1.6 (0.2)	4.5 (0.4)	3.5 (0.4)	6.1 (0.5)	2.9 (0.3)	7.3 (0.6)	164.3 (11.7)	4.6 (0.5)	0.2 (0.02)	30.4 (8.3)
Acacia debris	4.5 (0.0)	86.7 (20.4)	3224.3 (147.8)	3.0 (0.2)	8.2 (0.4)	6.0 (1)	6.5 (0.5)	1.8 (0.5)	5.0 (0.4)	11.3 (1.7)	8.5 (0.9)	1.0 (0.1)	52.9 (0.5)
Acacia debris and red clay	4.6 (0.0)	38 (6.2)	1840.7 (101.1)	2.0 (0.1)	4.7 (0.3)	3.7 (0.3)	4.8 (0.3)	1.4 (0.3)	3.4 (0.1)	32.7 (0.5)	5.6 (0.4)	0.5 (0.05)	29.9 (0.9)
Acacia debris and silica sand	4.7 (0.06)	56 (4)	1826.7 (196)	1.8 (0.2)	4.7 (0.5)	3.0 (0.05)	3.7 (0.5)	2.4 (0.3)	5.2 (0.4)	6.5 (0.2)	5.4 (0.7)	0.6 (0.1)	24.7 (1.3)

[1] Average

4.2.2. Survival of C. neoformans *on woody substrate*

Acacia mearnsii, commonly known as the black wattle, and *Eucalyptus camaldulensis*, commonly known as the river red gum, are exotic tree species originally imported from Australia, however both have become relatively common in Southern Africa. Thus, debris originating from these tree species was used as substrate for *C. neoformans* during this study. Also, *Eucalyptus* sp. have long been suspected of being the ecological niche of *C. gattii* (Ellis *et al*., 1990; Sorrell *et al*., 1997).

In an attempt to mimic low nutrient conditions when woody debris enters a soil environment, the debris of both tree species was mixed with red clay and silica sand. An incubation temperature of 26 °C was selected to test for the growth and survival of *C. neoformans* on woody substrates, since it corresponds more closely with environmental temperatures than temperatures associated with this yeast pathogen's mammalian hosts.

Representatives of *C. neoformans* var. *grubii*, VNI, MATα were selected for the experimentation since yeasts representing this genotype were found to be the most frequently isolated representative of *C. neoformans* world-wide (Meyer *et al*., 1999). The strains that were selected, namely *C. neoformans* var. *grubii* H99, CBS 10571 and MRC 8890, have originated from both environmental and clinical sources (see Appendix A).

All three strains representing *C. neoformans* var. *grubii* were able to grow and survive within the woody substrates up until the end of the incubation period (Figs 3 to 5). On the same substrate, the three strains showed similar growth curves, but the overall yeast numbers were significantly higher on substrates containing *A. mearnsii* debris than those containing *E. camaldulensis* debris. This may be as a result of the higher nitrogen levels present in the *A. mearnsii* woody material (Table 2). Ecologically, nitrogen is considered to an important limiting factor for many ecosystems (Blackburn, 1983), implicating that *A. mearnsii* could serve as a natural nitrogen reservoir.

Of the different woody substrates used in the study, woody debris together with red clay supported the smallest yeast population, while the growth curves obtained with this substrate was more erratic than on the other substrates (Fig 4). This could be due to the adsorption or sequestering of nutrients onto the clay (Marshall, 1980) or perhaps the adsorption of the yeast cells by the clay itself making

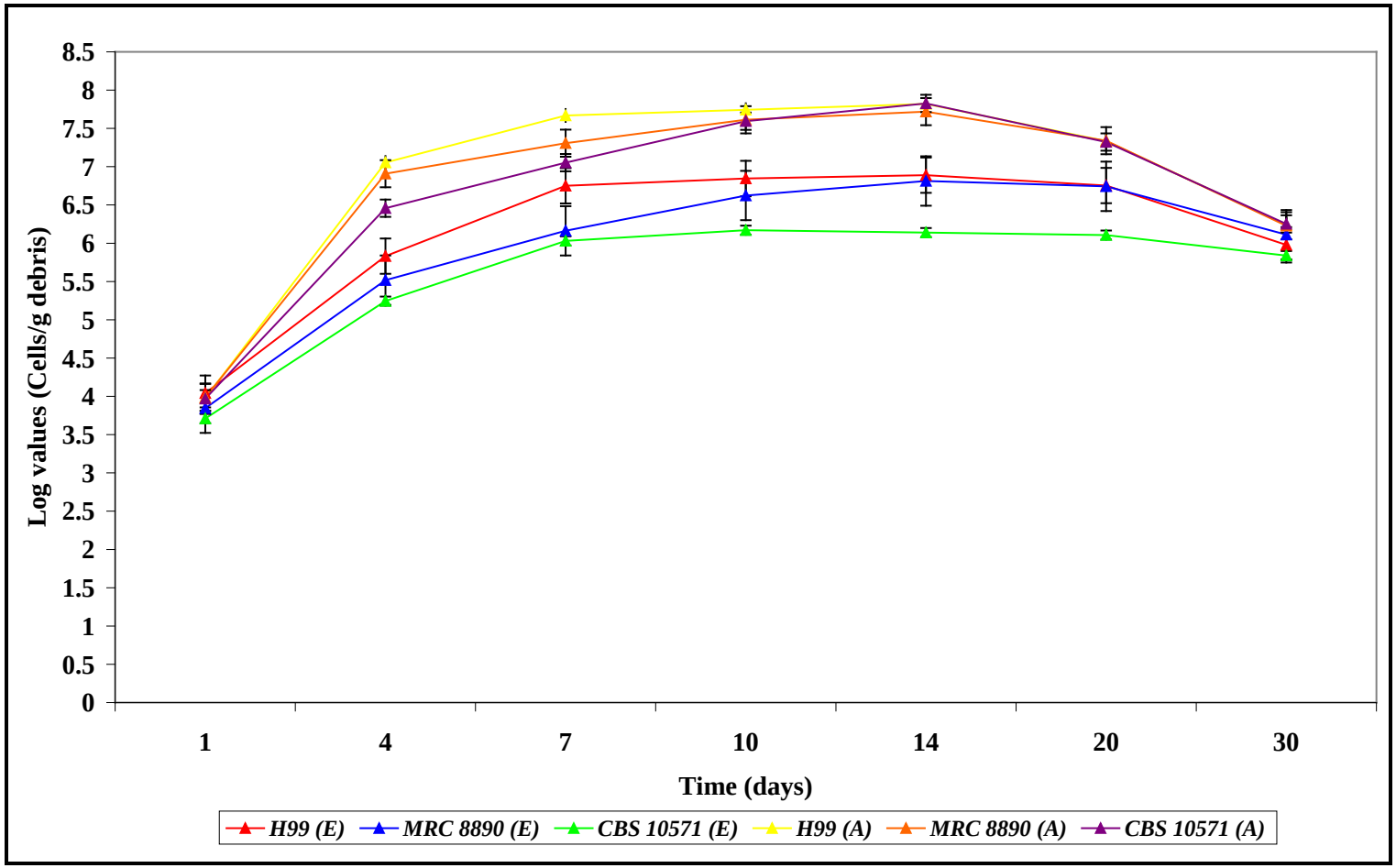

Figure 3 Growth curves of *C. neoformans* var. *grubii* H99; MRC 8890 and CBS 10571 in solid state cultures containing *E. camaldulensis* (E) or *A. mearnsii* (A) debris, at a moisture content equal to 100% field capacity, as substrate. Values represent the mean of three repetitions while the bars denote standard deviations.

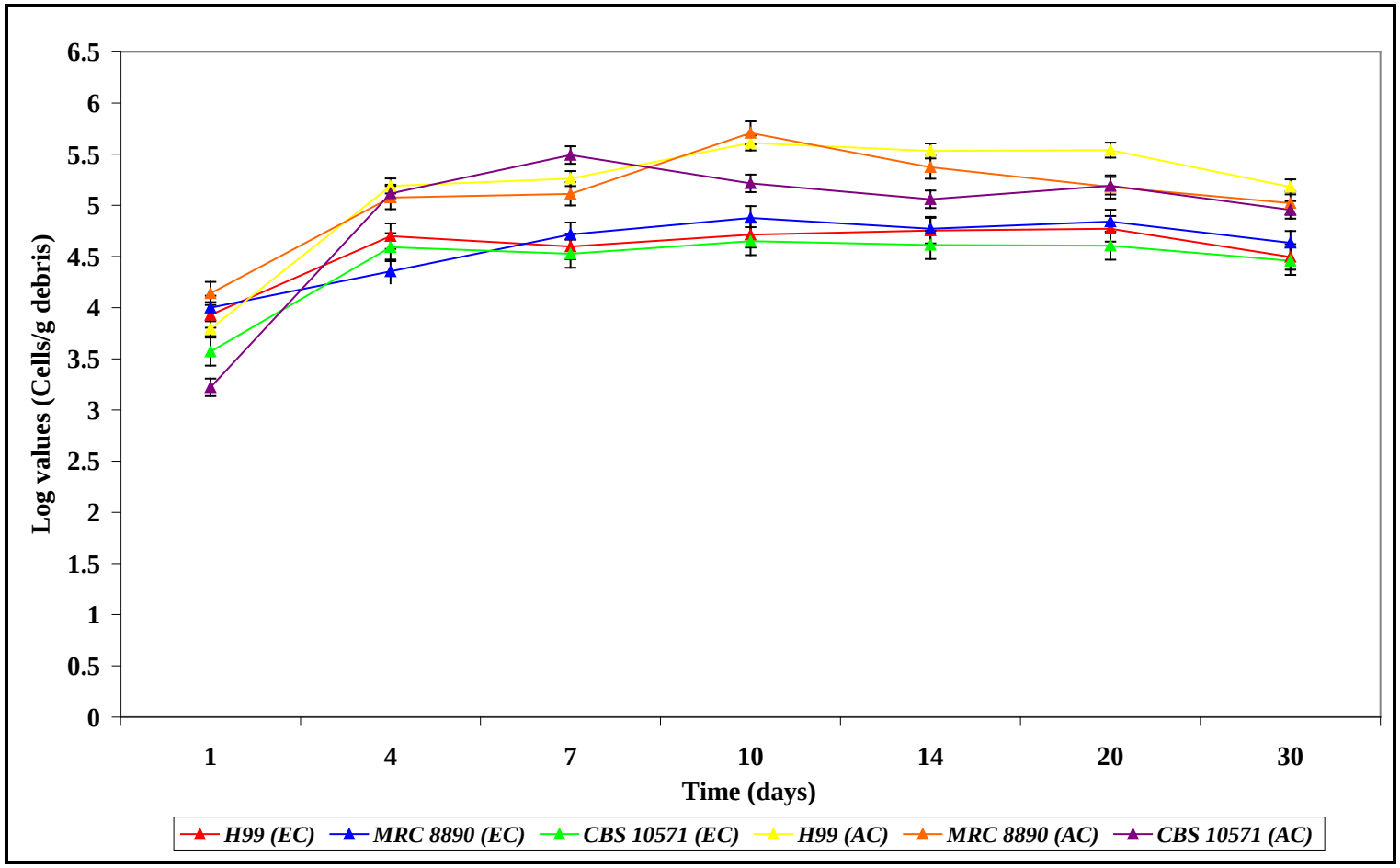

Figure 4 Growth curves of *C. neoformans* var. *grubii* H99; MRC 8890 and CBS 10571 in solid state cultures containing clay and either *E. camaldulensis* (EC) or *A. mearnsii* (AC) debris, at a moisture content equal to 100% field capacity, as substrate. Values represent the mean of three repetitions while the bars denote standard deviations.

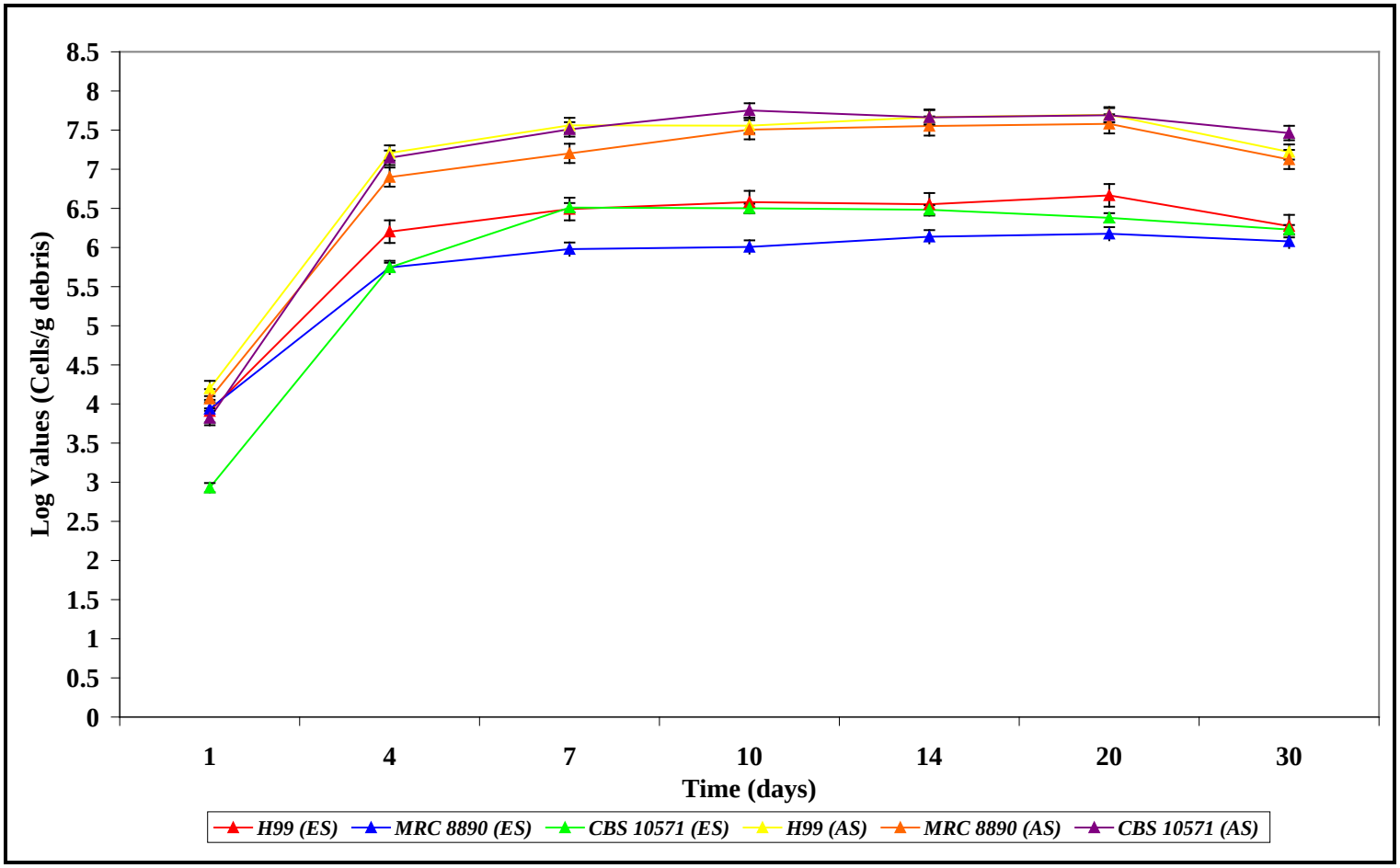

Figure 5 Growth curves of *C. neoformans* var. *grubii* H99; MRC 8890 and CBS 10571 in solid state cultures containing silica sand and either *E. camaldulensis* (ES) or *A. mearnsii* (AS) debris, at a moisture content equal to 100% field capacity, as substrate. Values represent the mean of three repetitions while the bars denote standard deviations.

high cell numbers difficult to sustain with nutrients only becoming available due to the death of other members of the yeast population.

The substrate comprising of woody debris and silica sand supported higher yeast populations than those containing clay (Figs. 4 and 5). Despite lower nutrient concentrations (Table 2), the yeasts remained viable for a longer period in substrates comprising of woody debris and silica sand than in substrates comprising solely of woody debris. The latter phenomenon may be as a result of the release of toxic components, such as tannins (Nathan *et al*., 2006; Smith *et al*., 2003) from the woody debris. Thus, substrates comprising of woody debris and silica sand seemed to be more favourable for the growth and survival of *C. neoformans* var. *grubii* and was used to study the interactions of this yeast and other selected microbes from the environment.

4.3. Survival of *C. neoformans* var. *grubii* on woody substrate in the presence of selected microbes

Although the isolation of soil and tree dwelling protists primarily associated with *C. neoformans* (data not shown) was successful, the introduction of these protistan predators into the woody substrate proved to be unsuccessful as we were unable to re-isolate any protista from substrate containing either *A. mearnsii* or *E. camaldulensis* debris. It was suggested that the woody substrate was limiting the accessibility of *C. neoformans* to the protists and were therefore unable to sustain a viable population on this limited nutrient source. The addition of bacteria isolated from enrichment cultures for protists that contained *A. mearnsii* and *E. camaldulensis* debris, would therefore serve as an alternative food source for the protista.

Two gram-negative bacteria were isolated and identified by means of 16S rDNA sequencing (Heyndrickx *et al*., 1996, see Appendix D) as *Pseudomonas fluorescens* (Fig 6) and an *Enterobacter* sp (Fig 7). Both genera are commonly associated with protists and fluorescently labelled *P. fluorescens* has often been used to monitor the presence of protista by serving as a growth medium (Ekelund *et al*., 1999). The addition of the bacteria to the woody substrate, however, proved to be unsuccessful and neither was able to sustain a viable population within the woody debris. As a result, the re-isolation of protists from the woody substrate proved to be unsuccessful once again.

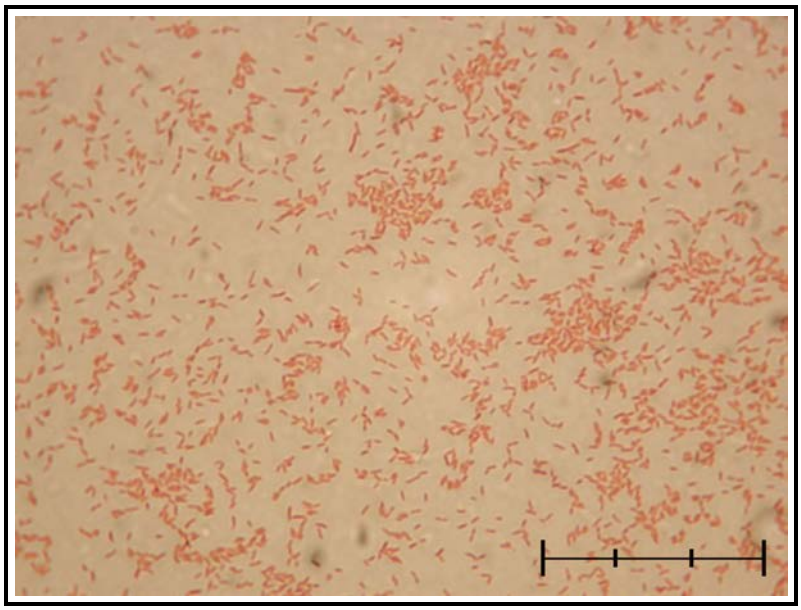

Figure 6 A gram stain prepared from a smear of the *P. fluorescens* isolate obtained from the protist enrichment cultures containing *A. mearnsii* debris viewed at 100x magnification (Nikon Eclipse E2000, Japan). Intervals of the scale bar represent 10 μM each.

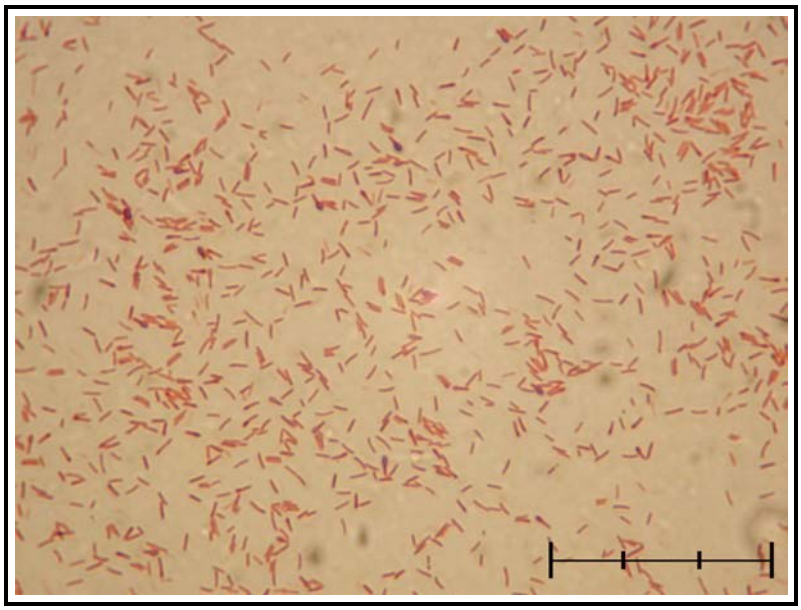

Figure 7 A gram stain prepared from a smear of the *Enterobacter* sp. isolate obtained from the protist enrichment cultures containing *E. camaldulensis* debris viewed at 100x magnification (Nikon Eclipse E2000, Japan). Intervals of the scale bar represent 10 μM each.

The inability of *P. fluorescens*, *Enterobacter* and the protists to survive within in the woody substrate could be a result of various compounds produced by both tree species. *Acacia mearnsii* and *E. camaldulensis* are both large producers of tannins and essential oils that are known for their anti-microbial and insecticidal activity (Nathan *et al*., 2006; Smith *et al*., 2003).

Tannins are water soluble polyphenolic compounds derived from a wide variety of plants and may protect the plants from pathogens, wood decay and herbivory (Smith *et al*., 2003). Although the exact anti-microbial action is not clear, it is thought that the tannins may insert or interact with the outer polar zone of the lipid bilayer causing disruption of the membrane structure and therefore cell leakage. In addition, condensed tannins such as those originating from *A. mearnsii*, have been shown to be toxic towards *Escherichia coli* primarily due to the generation of hydrogen peroxide (Smith *et al*., 2003).

The Eucalypt species are perhaps better known for their production of essential oils, specifically eucalypt oil. The exact anti-microbial mechanism is also not clearly understood, but it has been suggested that membrane disruption by the lipophilic components is involved (Schelz *et al*., 2006). Recently, eucalypt oil as well as tannins extracted from *E. camaldulensis* has been shown to posses bacterial and fungicidal activity towards *Bacillus subtilis*, *Staphylococcus aureus*, *Microsporium gypseum*, *Trichophyton mentagrphytes* and *Candida albicans* (Falahati *et al*., 2005; Babayi *et al*., 2004). Interestingly, eucalypt oil was found to be an effective mosquito-cide by increasing larval mortality of the malaria vector *Anopheles stephensi* (Nathan *et al*., 2006).

It should however be mentioned that although the bacteria were isolated from enrichment cultures for protists containing *A. mearnsii* or *E. camaldulensis* debris, the subsequent sterilization of the woody substrates by means of autoclaving may have allowed a crude water extraction of these tannins that would limit the growth and survival of both the bacteria and protistan species. Membrane delimited microbes, such as gram negative bacteria and protists, may therefore be especially vulnerable in environments containing large quantities of *A. mearnsii* or *E. camaldulensis* debris.

4.4 The ability of *C. neoformans* to fruit on woody debris

As *C. neoformans* var. *grubii* was capable of growth on substrates containing woody debris, we examined the hypothesis that this yeast is also able to fruit on media containing *A. mearnsii* and *E. camaldulensis* debris. All the *C. neoformans* strains (see Appendix A) were crossed with one of three reference strains, namely *C. neoformans* var. *neoformans* JEC 20 (*a*/D); *C. neoformans* var. *neoformans* JEC 21 (α/D) and *C. neoformans* var. *grubii* CBS 9172 (*a*/A). Finally one plate was left as single inoculums in order to determine if the *C. neoformans* strains were capable of monokaryotic fruiting on this woody debris.

A total of 19 % and 6 % of the *C. neoformans* strains were able to mate when crossed on *A. mearnsii* (Table 3) and *E. camaldulensis* (Table 4) debris, respectively. This may be the first observation of the hyphal phase of *C. neoformans* on a medium containing woody debris as sole nutrient source. Only *C. neoformans* var. *grubii* CBS 10574, an environmental strain isolated from soil of the North West province of South Africa, demonstrated limited monokaryotic fruiting when cultured on the woody debris. This low frequency of monokaryotic fruiting among the isolates could be a result of high tannin levels or perhaps the media provided enough nutrients to allow for normal asexual reproduction via budding.

Strains showing no fruiting structures were capable of yeast-like growth on the woody debris displaying brown, “jelly-like” macroscopic appearance, characteristic of the order *Tremellales* (Fig 8). When hyphal development occurred, it originated from the edges of the colony and spread across the media (Fig 9). In some cases, hyphal patches developed in the centre of the yeast colony, although lateral growth remained limited. Interestingly, older hyphae developed a brown pigmentation (Fig 10). This could be due to the production and incorporation of melanin into the cell wall or this colouration could be contributed to the pigmentation of the woody debris media. The formation of basidia and basidiospores was confirmed using a compound microscope (Fig 11 and 12).

Table 3 Fertile *C. neoformans* strains when crossed with suitable reference strains on media containing the woody debris of *A. mearnsii*.

Ref Number	Genotype	Mating type	Crossing on *A. mearnsii* media			
			CBS9172 (*a*/A)	JEC20 (*a*/D)	JEC21 (α/D)	Haploid fruiting
H99	VNI	MATα	+	+	-	-
CBS 10571	VNI	MATα	-	+	-	-
CBS 10573	VNI	MATα	-	+	-	-
CBS 10574	VNI	MATα	+	+	-	+ *
MRC 8891	VNI	MATα	-	+	-	-
MRC 8883	VNI	MATα	-	+	-	-
MRC 8857	VNII	MATα	-	+	-	-
MRC 8858	VNII	MATα	-	+	-	-
MRC 8865	VNI	MATα	-	+	-	-

* Limited hyphal growth observed under the microscope (Nikon SMZ-10A, Japan)

Table 4 Fertile *C. neoformans* strains when crossed with suitable reference strains on media containing the woody debris of *E. camaldulensis.*

Ref Number	Genotype	Mating type	Crossing on *E. camaldulensis* media			
			CBS9172 (*a*/A)	JEC20 (*a*/D)	JEC21 (α/D)	Haploid fruiting
CBS 10574	VNI	MATα	-	+ *	-	+ *
MRC 8865	VNI	MATα	-	+ *	-	-

* Limited hyphal growth observed under the microscope (Nikon SMZ-10A, Japan)

The majority of mating occurred on the media containing *A. mearnsii* as nutrient source, indicating that the tree species may impact on the mating frequency of *C. neoformans*. Similar observations have been made for *C. gattii*, a closely related species of *C. neoformans*. The former species show a strong ecological association with certain Eucalypt species within Australia, particularly *E. camaldulensis* (Ellis *et al*., 1990). However, despite reported isolation of both mating types from a single

tree, the population structure seemed to remain clonal and showed no signs of recombination (Halliday *et al*., 2003). Interestingly, different trees harboured genetically distinct populations of *C. gattii* indicating that there was no dispersal of the pathogen between trees.

All fertile strains identified on the woody debris were *C. neoformans* var. *grubii*, however, only two of these fertile strains mated with the reference strain of the same serotype, namely *C. neoformans* var. *grubii* CBS 9172. This inability or delayed mating could be as a result of continual culturing of the reference strain *C. neoformans* var. *grubii* CBS 9172. Alternatively, the impaired mating between *C. neoformans* var. *grubii* strains of opposite mating types may give an indication as to the mating type bias seen within environmental isolates. Within environmental settings, the mating type bias reaches ratios as high as 40:1 (Halliday *et al*., 1999), although rare cases of equal ratios have been reported for *C. neoformans* in Botswana (Litvintseva *et al*., 2006) as well as the closely related species *C. gattii* in Australia (Halliday *et al*., 1999).

Figure 8 Mating between *C. neoformans* var. *grubii* CBS 10571 and CBS 9172 when crossed on *A. mearnsii* produced a “jelly-like” macroscopic appearance characteristic of the order Tremellales (Nikon SMZ-10A, Japan).

Figure 9 Mating between *C. neoformans* var. *grubii* strains H99 and CBS 9712 when crossed on *A. mearnsii* (Nikon SMZ-10A, Japan).

Figure 10 Pigmented hyphae produced by mating between *C. neoformans* var. *grubii* MRC 8857 and *C. neoformans* var. *neoformans* JEC 20 when crossed on *A. mearnsii* (Nikon SMZ-10A, Japan).

Figure 11 The production of basidiospores and basidia during mating of *C. neoformans* var. *grubii* CBS 10573 and CBS 9172 when crossed on *A. mearnsii* viewed at 400x magnification (Nikon Eclipse E2000, Japan). Intervals of the scale bar represent 10 μM each.

Finally, only one strain, *C. neoformans* var. *grubii* CBS 10574, displayed limited monokaryotic fruiting. Monokaryotic or haploid fruiting is thought to be induced during certain stress conditions, particularly nutrient starvation and nitrogen limitation (McClelland *et al*., 2003; Tscharke *et al*., 2003; Wickes *et al*., 1996) and is distinguished from dikaryotic fruiting primarily by the development of un-fused clamp connections (Wickes *et al*., 1996). The monokaryotic fruiting of *C. neoformans* var. *grubii* CBS 10574 on *A. mearnsii* was unexpected as this tree species is known to be nitrogen fixing, maintaining high levels of nitrogen. This may indicate that a number of factors, not simply nitrogen limitation, are implicated during the monokaryotic fruiting of *C. neoformans*.

Figure 12 The production of basidiospores and basidia during mating of *C. neoformans* var. *grubii* CBS 10573 and *C. neoformans* var. *neoformans* JEC 20 when crossed on *A. mearnsii* viewed at 1000x magnification (Nikon Eclipse E2000, Japan). Intervals of the scale bar represent 10 μM each.

4.5. Fruiting of *C. neoformans* on standard media

As a control for the mating studies conducted with media containing woody debris, the fertility of all *C. neoformans* strains (See Appendix A) was tested on standard V8 juice and yeast carbon base (YCB) agar. A total of 44 % and 72 % of all *C. neoformans* strains tested displayed fertility on V8 juice (Table 5) and YCB (Table 6) agar respectively. Once again *C. neoformans* var. *grubii* strains mated more readily with the reference strain of a different serotype, *C. neoformans* var. *neoformans* JEC 20 (*a*/D) when crossed on V8 juice agar. An increase in mating frequency between strains of the same serotype (serotype A) was noted when strains were crossed on YCB agar. However, hyphae developed at a much slower rate and formed tuffs within the edges of the colony as opposed to the lateral growth noted between crosses of opposite serotypes.

This increase in mating frequency may be as a result of nutrient limitations, particularly nitrogen as this has been associated with monokaryotic fruiting of

C. neoformans. However, only one strain, namely *C. neoformans* var. *neoformans* MRC 8856, developed monokaryotic hyphae (Fig 13) and unlike serotype A, serotype D is regarded as being a vigorous monokaryotic fruiter (Tscharke *et al*., 2003)

Table 5 Fertile *C. neoformans* strains when crossed with suitable reference strains on V8 juice agar.

Ref Number	**Genotype**	**Mating type**	**Crossing on V8 juice agar**			
			CBS 9172 (*a*/A)	**JEC 20 (*a*/D)**	**JEC 21 (α/D)**	**Haploid fruiting**
H99	VNI	MATα	-	+	-	-
CBS 10571	VNI	MATα	+	+	-	-
CBS 10572	VNI	MATα	-	+	-	-
CBS 10573	VNI	MATα	+	+	-	-
MRC 8888	VNI	MATα	+	+	-	-
MRC 8890	VNI	MATα	-	+	-	-
MRC 8879	VNI	MATα	+	+	-	-
MRC 8880	VNII	MATα	-	+	-	-
MRC 8883	VNI	MATα	+	-	-	-
MRC 8857	VNII	MATα	-	+	-	-
MRC 8861	VNI	MATα	-	+	-	-
MRC 8864	VNI	MATα	-	+	-	-
MRC 8865	VNI	MATα	-	+	-	-
MRC 8866	VNI	MATα	-	+	-	-
MRC 8867	VNI	MATα	+	-	-	-
MRC 8868	VNII	MATα	-	+	-	-

Table 6 Fertile *C. neoformans* strains when crossed with suitable reference strains on YCB agar.

Ref Number	Genotype	Mating type	Crossing on YCB media			
			CBS 9172 (*a*/A)	JEC 20 (*a*/D)	JEC 21 (α/D)	Haploid fruiting
H99	VNI	MATα	+	+	-	-
CBS 132	VNIV	MATα	+ *	-	-	-
CBS 10571	VNI	MATα	+	+	-	-
CBS 10572	VNI	MATα	-	+	-	-
CBS 10573	VNI	MATα	+	+	-	-
CBS 10574	VNI	MATα	-	+	-	-
MRC 8887	VNI	MATα	+ *	+	-	-
MRC 8888	VNI	MATα	-	+	-	-
MRC 8890	VNI	MATα	+	+	-	-
MRC 8891	VNI	MATα	-	+	-	-
MRC 8879	VNI	MATα	+	+	-	-
MRC 8880	VNII	MATα	+ *	+	-	-
MRC 8882	VNI	MATα	+ *	-	-	-
MRC 8884	VNII	MATα	+ *	+	-	-
MRC 8885	VNI	MATα	+ *	-	-	-
MRC 8855	VNII	MATα	+ *	-	-	-
MRC 8856	VNIV	MATα	+	+	-	+
MRC 8857	VNII	MATα	+	+	-	-
MRC 8858	VNII	MATα	+ *	-	-	-
MRC 8859	VNI	MATα	+ *	-	-	-
MRC 8860	VNI	MATα	+ *	-	-	-
MRC 8861	VNI	MATα	+ *	-	-	-
MRC 8863	VNII	MATα	+ *	-	-	-

*Limited hyphal growth observed under the microscope (Nikon SMZ-10A, Japan)

Table 6 (cont) Fertile *C. neoformans* strains when crossed with suitable reference strains on YCB agar.

Ref Number	Genotype	Mating type	Crossing on YCB media			
			CBS 9172 (*a*/A)	JEC 20 (*a*/D)	JEC 21 (α/D)	Haploid fruiting
MRC 8864	VNI	MATα	+ *	-	-	-
MRC 8865	VNI	MATα	-	+	-	-
MRC 8866	VNI	MATα	+ *	-	-	-
MRC 8867	VNI	MATα	+ *	-	-	-
MRC 8868	VNI	MATα	+	-	-	-

*Limited hyphal growth observed under the microscope (Nikon SMZ-10A, Japan)

Figure 13 Monokaryotic fruiting observed for *C. neoformans* var. *neoformans* MRC 8856 when cultured on yeast carbon base (YCB) a nitrogen limited medium. (Nikon SMZ-10A, Japan).

5. CONCLUSIONS

All strains of the opportunistic human pathogen, *C. neoformans* var. *grubii*, showed cellulase activity in plate assays. The latter is an essential cellulolytic enzyme required for growth within a woody environment. In addition, in contrast to protistan

predators and gram negative bacteria, representative strains of *C. neoformans* var. *grubii* were capable of growth and survival when cultured on woody debris as sole substrate. The extent of growth however, seemed to depend on the tree species from which the woody debris originated. Some strains were able to mate on a medium containing the woody debris as sole nutrient source however; the frequency of this mating among the strains again seemed to depend on the tree species from which the woody debris originated. Nevertheless, it is obvious that *C. neoformans* var. *grubii* is capable of producing all its ontogenic stages when cultured on woody debris.

This may be the first reported observation of the hyphal phase of *C. neoformans* when cultured on woody debris, the suspected habitat of this yeast pathogen. Further studies involving the interactions of *C. neoformans* and other naturally occurring microbes need to be conducted in order to understand the mechanism of survival and dispersal within the true ecological niche of this fungal pathogen

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6. LITERATURE CITED

Abbott T. S. (1985). In: Abbott T. S. (Ed.) *Soil testing service: Methods and Interpretation*, NSW Department of Agriculture.

Aksenov S. I., Babyeva I. P., Golubev V. I. (1973). On the mechanism of adaptation of micro-organisms to conditions of extreme low humidity. *Life Sci. Space Res.* **11:** 55-61.

Ausubel F. M., Brent R., Kingston R. E., Moore D. D., Seidman J. D., Smith J. A., Struhl K. (Eds.) (1989). *Current Protocols in Molecular Biology.* **2:** Wiley, New York.

Babayi H., Kolo I., Okogun J. I., Ijah U. J. J. (2004). The antimicrobial activities of methanolic extracts of *Eucalyptus camaldulensis* and *Terminalia catappa* against some pathogenic microorganisms. *Biokem.* **16, 2:** 106-111.

Barluzzi, R., A. Brozzetti, G. Mariucci, M. Tantucci, R. G. Neglia, F. Bistoni, and E. Blasi. (2000). Establishment of protective immunity against cerebral cryptococcosis by means of an avirulent, nonmelanogenic *Cryptococcus neoformans* strain. *J. Neuroimmunol*. **109:** 75–86.

Beyers C. P., Coetzer F. J. (1971). Effect of concentration, pH and time on the properties of di-ammonium EDTA as a multiple soil extractant. *Agrochemophysica.* **3:** 49-54.

Blackburn T. H. (1983). The micribial nitrogen cycle. In: Krumbein C. W. E. (Ed.) *Microbial Geochemistry*, Blackwell, Oxford, England, pp. 63-89.

Bremner J. M. (1965). Inorganic forms of nitrogen. In: Black C. A. (Ed.) *Methods of Soil Analysis, Part 2.* American Society of Agronomy, Madison, W.I. pp. 1179-1237.

Briones Jr. A. M., Reichardt W. (1999). Estimating microbial population counts by 'most probable number' using Microsoft Excel®. *J. Microbiol. Meth.* **35:** 157-161.

British Columbia, Ministry of Agriculture, Food and Fisheries. Resource Management Branch (2002). Soil Water Storage Capacity and Available Soil Moisture. *Water Conservation FACTSHEET* (http://www.agf.gov.bc.ca/resmgmt/publist/600series/619000-1.pdf).

Butler T., Alcalde M., Sieber V., Meinhold P., Schlachtbauer C., Arnold F. H. (2003). Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. *Appl. Environ. Microbiol*. **69, 2:** 987-995.

Bunting L. A., Neilson J. B., Bulmer G. S. (1979). *Cryptococcus neoformans*: gastronomic delight of a soil ameba. *Sabouraudia.* **17, 3:** 225-232.

Chen L., Pirofski L., Casadevall A. (1997). Extracellular proteins of *Cryptococcus neoformans* and host antibody response. *Infect. Immun.* **65, 7:** 2599-2605.

Chory J., Pollard J. D. Jr. (1999). Resolution and recovery of small DNA fragments. In: Chory J., Pollard J. D. Jr. (Eds.) *Current Protocols in Molecular Biology.* **2:** 2.7.1- 2.7.8.

Cogliati M., Esposto M. C., Clarke D. L., Wickes B. L., Viviani M. A. (2001). Origin of *Cryptococcus neoformans* var. *neoformans* diploid strains. *J. Clin. Microbiol*. **39, 11:** 3889-3894.

Decho A.W. (1990) Microbial exopolymer secretions in ocean environments: Their role(s) in food webs and marine processes. *Oceanogr. Mar. Biol. Annu. Rev.* **28:** 73 – 153.

De Kokker T. H., Zhao J., Allsop S. F., Janse B. J. H. (2000). Isolation and enzymatic characterization of South African white rot fungi. *Mycol. Res*. **104:** 820-824.

Doering T. L., Nosanchuk J. D., Roberts W. K., Casadevall A. (1999). Melanin as a potential cryptococcal defense against microbiocidal proteins. *Med. Mycol.* **37:** 175-181.

Doll E. C., Lucas R. E. (1973). Testing soils for potassium, calcium and magnesium. In: Walsh L. M., Beatons J. D. (Eds.) *Soil testing and plant analysis.* Science Society of America, Madison, W.I. pp. 133-152.

Ekelund F (1998). Enumeration and abundance of mycophagous protozoa in soil, with special emphasis on heterotrophic flagellates. *Soil. Biol. Biochem.* **30, 10/11:** 1343-1347.

Ekelund F., Christensen S., Ronn R., Buhl E., Jacobson C. S. (1999). An automated technique for most-probable-number (MPN) analysis of densities of phagotrophic protists with *lux* AB labelled bacteria as growth medium. *J. Microbiol. Meth.* **39:** 177-182.

Ellis D. H., Pfeiffer T. J. (1990). Natural habitat of *Cryptococcus neoformans* var. *gattii*. *J. Clin. Microbiol.* **28, 7:** 1642-1644.

Falahati M., Tabrizib N. O., Jahaniani F. (2005). Anti dermatophyte activities of *Eucalyptus camaldulensis* in comparison with Griseofulvin. *Iranian J. Pharmacol. Therapeut*. **4, 2:** 80-83.

Farkas V. M., Liskova, Bielly P. (1985). Novel media for detection of microbial producers of cellulase and xylanase. *FEMS Microbiol. Lett*. **28:** 137-140.

Feldmesser M., Kress Y., Novikoff P., Casadevall A. (2000). *Cryptococcus neoformans* is a facultative intracellular pathogen in murine pulmonary infection. *Infect. Immun.* **68:** 4225–4237.

Fertilizer Society of South Africa. (1974). Manual of soil analysis methods. FSSA Publication **No. 37**. Fertilizer Society of South Africa, Lynnewood Ridge.

Forrester D. I., Schortemeyer M., Stock W. D., Bauhus J., Khanna P. K., Cowie A. L. (2007). Assessing nitrogen fixation in mixed- and single-species plantations of *Eucalyptus globulus* and *Acacia mearnsii*. *Tree Physiol.* **27:** 1319–1328.

Fraser J. A., Subaran R. L., Nichols C. B., Heitman J. (2003). Recapitulation of the sexual cycle of the primary fungal pathogen *Cryptococcus neoformans* var. *gattii*: implications for an outbreak on Vancouver Island, Canada. *Eukaryot. Cell*. **2, 5:** 1036-1045.

Garcia-Rivera J., Casadevall A. (2001). Melanization of *Cryptococcus neoformans* reduces its susceptibility to the antimicrobial effects of silver nitrate. *Med. Mycol.* **39:** 353-357.

Hall J., Hazlewood G. P., Huskisson N. S., Durrant A. J., Gilbert H. J. (1989). Conserved serine-rich sequences in xylanase and cellulase from *Pseudomonas*

fluorescens subspecies *cellulosa*: internal signal sequence and unusual protein processing. *Mol. Microbiol.* **3, 9:** 1211–1219.

Halliday C., Bui T., Krockenberger M., Malik R., Ellis D., Carter D. (1999). Presence of α and *a* mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gatti* strains from Australia. *J. Clin. Microbiol.* **37,** 2920-2926.

Heyndrickx M., Vauterin L., Vandamme P., Kersters K., De Vos P. (1996). Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *J. Microbiol. Meth.* **26:** 247-259.

Kwon-Chung K. J. (1998). *Filobasidiella* Kwon-Chung. In: Kurtzman C. P., Fell J. W. (Eds.) *The Yeasts, a Taxonomic Study*, Elsevier Science BV, Amsterdam pp. 656-662.

Kwon-Chung K. J., Bennett, J. E. (1984). Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. *Am. J. Epidemiol.* **120:** 123-130.

Küskinen L., L. Rättä M., Kruus K. (2004). Screening for novel laccase-producing microbes. *J. Appl. Microbiol.* **97, 3:** 640–646.

Lazéra M. S., Pires F. D. A., Camillo-Coura L., Nishikawa M. M., Bezerra C. C. F., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *J. Med. Vet. Mycol.* **34:** 127-131.

Lengeler K. B., Fox D. S., Fraser J. A., Allen A., Forrester K., Dietrich F. S., Heitman J (2002). Mating-type locus of *Cryptococcus neoformans*: A step in the evolution of sex chromosomes. *Eukaryot. Cell.* **1, 5:** 704-718.

Loftus B. J., Fung E., Roncaglia P., Rowley D., Amedeo P., Bruno D., Vamathevan J., Miranda M., Anderson I. J., Fraser J. A., Allen J. E., Bosdet I. E., Brent M. R., Chiu R., Doering T. L., Donlin M. J., D'Souza C. A., Fox D. S., Grinberg V., Fu J., Fukushima M., Haas B. J., Huang J. C., Janbon G., Jones S. J., Koo H. L., Krzywinski M. I., Kwon-Chung J. K., Lengeler K. B., Maiti R., Marra M. A., Marra R. E., Mathewson C. A., Mitchell T. G., Pertea M., Riggs F. R., Salzberg S. L., Schein J. E., Shvartsbeyn A., Shin H., Shumway M., Specht C. A., Suh B. B., Tenney A., Utterback T. R., Wickes B L., Wortman J R., Wye N. H., Kronstad J. W., Lodge J. K., Heitman J., Davis R. W., Fraser C. M., Hyman R. W. (2005). The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans. Science.* **307:** 1321-1324.

Marshall K. C. (1980). Adsorption of microorganisms to soils and sediments. In: Bitton G., Marshall K. C. (Eds.) *Adsorption of microorganisms to surfaces*, Wiley, New York, pp 317-329.

McClelland C. M., Chang Y. C., Varma A., Kwon-Chung K. J. (2003). Uniqueness of the mating system in *Cryptococcus neoformans*. *Trends Microbiol.* **12, 5:** 208-212.

Min K. L., Kim Y. H., Kim Y. W., Jung H. S., Hah Y. C. (2001). Characterization of a novel laccase produced by the wood-rotting fungus *Phellinus ribis. Arch. Biochem. Biophys.* **392, 2:** 279-286.

Nathan S. S. (2006). The use of *Eucalyptus tereticornis* Sm. (Myrtaceae) oil (leaf extract) as a natural larvicidal agent against the malaria vector *Anopheles stephensi* Liston (Diptera: Culicidae). *Bioresource Tech.* **98, 9:** 1856-1860.

Nelson D. W., Sommers L. E. (1982). Total carbon, organic carbon and organic matter. In: Page A. L. (Ed.) *Methods of Soil Analysis, Part 2*, American Society of Agronomy, Madison, W.I. p 199.

Prichard T. L. (2005) Soil moisture measurement technology. University of California Davis. (http://ucce.ucdavis.edu/files/filelibrary/40/975.pdf).

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia.* **151:** 63-69.

Rosas A. L, Casadevall A. (1997). Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold. *FEMS Microbiol. Lett.* **153:** 265-272.

Rosas A. L., Casadevall A. (2001). Melanin decreases the susceptibility of *Cryptococcus neoformans* to enzymatic degradation. *Mycopathologia.* **151:** 53-56.

Schelz Z., Molnar J., Hohmann J (2006). Antimicrobial and antiplasmid activities of essential oils. *Fitoterapia.* **77:** 279-285.

Smith A. H., ImLay J. A., Mackie R. I. (2003). Increasing the oxidative stress response allows *Escherichia coli* to overcome inhibitory effects of condensed tannins. *Appl. Environ. Microbiol.* **69, 6:** 3406-3411.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans*. *Rev. Iberoam. Microbiol*. **14:** 42-43.

Steenbergen J. N., Shuman H. A., Casadevall A. (2001). *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proc. Natl. Acad. Sci. USA.* **18:** 15245-15250.

Thomas G.W., and Peaslee D.E. (1973). Testing for soil phosphorus. In: Walsh L. M., Beatons J. D. (Eds) *Soil Testing and Plant Analysis*, Soil Science Society of America, Madison, W.I. pp. 15-122.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout, T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Med. Mycol.* **41:** 383-390.

Tscharke R. L., Lazéra M., Chang Y. C., Wickes B. L., Kwon-Chung K. J. (2003). Haploid fruiting in Cryptococcus neoformans is not mating type α-specific. *Fungal Genet. Biol.* **39:** 230-237.

Tucker S. C., Casadevall A. (2002). Replication of *Cryptococcus neoformans* in macrophages is accompanied by phagosomal permeabilization and accumulation of vesicles containing polysaccharide in the cytoplasm, *Proc. Natl. Acad. Sci. USA.* **99:** 3165–3170.

Vidotto V., Koga-Ito C. Y., Canella D., Sinicco A., Perri G. D., Aoki S., Ito-Kuwa S. (2000). Extracellular activity in *Cryptococcus neoformans* strains isolated from AIDS patients and from environmental sources. *Rev. Iberoam. Micol.* **17:** 14-19.

Wang Y., Casadevall A. (1994). Decreased susceptibility of melanized *Cryptococcus neoformans* to UV light. *Appl. Environ. Microbiol.* **60:** 3864-3866.

Wickes B. L., Mayorga M. E., Edman U., Edman J. C. (1996). Dimorphism and haploid fruiting in *Cryptococcus neoformans*: Association with the α-mating type. *Microbiol.* **93:** 7327-7331.

Williamson P., R. (1997). Laccase and melanin in the pathogenesis of *Cryptococcus neoformans, Front. Bioscience*. **2:** 99–107.

Yarrow D. (1998). Methods for isolation, maintenance and identification of yeasts. In: Kurtzman C. P., Fell J. W. (Eds.) *The Yeasts, a Taxonomic Study,* Amsterdam: Elsevier., pp. 77-98.

Zhu X., Gibbons J., Garcia-Rivera J., Casadevall A., Williamson P. R. (2001). Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. *Infect. Immun.* **69, 9:** 5589-5596.

Chapter 4

General Conclusions and Future Research

1. GENERAL CONCLUSIONS AND FUTURE RESEARCH

Cryptococcus neoformans (Sanfelice) Vuillemin is an opportunistic fungal pathogen responsible for causing meningitis predominantly in immuno-compromised individuals (Casadevall *et al*., 2003; Franzot *et al*., 1998; Mitchell and Perfect, 1995), particularly those suffering from human immuno virus (HIV) and acquired immuno-deficiency syndrome (AIDS). The incidence of infection, also known as cryptococcosis, among these individuals is estimated at approximately 10 % (Chuck and Sande, 1989).

From a global medical perspective, *C. neoformans* var. *grubii* (Serotype A, MATα) is responsible for approximately 90 % of all cryptococcosis infections (Mitchell and Perfect, 1995). Although representatives of this variant can be grouped into two different genotypes, namely VNI and VNII, the genotype VNI appears to dominant, having a worldwide distribution (Meyer *et al*., 1999). This genotype has been isolated from 78 % of global AIDS related cryptococcal infections. Similarly, it was isolated from 57 % and 88 % of AIDS related cryptococcal infections in the provinces of Gauteng and Kwa-Zulu Natal, South Africa, respectively.

Results obtained during this study for the Gauteng, North West and Western Cape provinces demonstrate similar trends. All strains were identified as being mating type alpha (MATα) with the majority of strains representing genotype VNI (75.6 %) and seven strains representing genotype VNII (21.2 %) and only one strain representing genotype VNIV (3 %). Further research will have to be conducted in order to determine the prevalence of genotypes and mating types of *C. neoformans* strains from the remaining South African provinces. This would have to include large scale environmental sampling projects throughout the provinces of South Africa. We recommend however, that sample type be limited to decaying wood or woody debris, the perceived natural habitat of this yeast pathogen.

Indeed, *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* have been isolated from a variety of environmental sources including soil, vegetative debris and particularly decaying wood and the hollows of trees (Trilles *et al*., 2003; Randhawa *et al*., 2001; Lazéra et *al*., 1996). The latter source appears to be promising with regards to locating the ecological niche of *C. neoformans* as the pathogen

produces a laccase enzyme known to be a potent virulence factor in addition to its ligninolytic activity (Min *et al*., 2001). The laccase enzyme, coupled to the recent discovery of a cryptococcal cellulase (Loftus *et al*., 2005), further links *C. neoformans* to a potentially woody ecological niche.

Our research firstly screened for the presence of wood degrading enzymes amongst the *C. neoformans* strains originating from South Africa. All strains tested positive for cellulase activity, 6 % of stains tested positive for laccase, activity while none were capable of degrading xylan. A greater number of clinical and environmental strains of *C. neoformans* need to be included in further experimentation in order to determine the full physiological capabilities of these yeasts. Strains should also be studied for the activity of other enzymes, such that of urease, proteinase and phospholipase.

During this study, we tested the hypothesis that *C. neoformans* is capable of growth when cultured on woody debris. *C. neoformans* var. *grubii* (serotype A, MATα, VNI) strains originating from both clinical and environmental sources were capable of growth over the experimental period when cultured solely on a woody substrate. Similar experimentation should be conducted in order to determine if the remaining genotypes, namely VNII VNIII and VNIV, as well as the opposite mating type (MAT*a*), performs similarly under these conditions. Interestingly, the type of woody debris selected, namely *Acacia mearnsii* and *Eucalyptus camaldulensis*, appeared to impact on the growth of *C. neoformans*. The incorporation of different tree species into the experimentation may give a clearer indication as to whether *C. neoformans* displays any preference to a particular tree species.

Our research was also able to demonstrate that *C. neoformans* is capable of both dikaryotic and monokaryotic fruiting when cultured on woody debris. This may be the first reported observation of the hyphal phase of *C. neoformans* when cultured on this substrate. Once again, a greater number of clinical and environmental *C. neoformans* strains, as well as same sex mating crosses, need to be included during further experimentation. This will help determine the fertility of the South African *C. neoformans* isolates. The inclusion of different tree species as a mating substrate may also be done to detect any environmental associations of *C. neoformans*.

Although not examined during this study, the antibiotic resistance of the

C. neoformans strains towards the prescribed anti-fungal agents needs to be evaluated. Antibiotic resistance amongst *C. neoformans* strains is relativity rare, however; the prolonged use of fluconazole and itraconazole within our AIDS populations warrants continual surveillance in order to detect the emergence of less susceptible populations of *C. neoformans*.

2. UNANSWERED QUESTIONS

A number of unanswered questions remain concerning *C. neoformans*. Firstly, the true ecological niche of this pathogen remains undiscovered. Although *C. neoformans* has been isolated from a number of environmental sources, it shows no ecological associations.

The mechanisms of pathogenesis also remain largely unresolved. Firstly, the infectious particles are regarded as being either desiccated yeast cells or aerolized basidiospores (Sorrell *et al*., 1997). The ability of *C. neoformans* to traverse the blood-brain barrier is also unclear.

In terms of characterization, the question concerning the predominance of the MATα mating type has not yet been resolved. Coupled to this is the large clonality observed amongst *C. neoformans* populations, despite the presence of both mating types (Trilles *et al*., 2003; Halliday *et al*., 1999).

Finally, with the completion of the *C. neoformans* genome sequencing project (Loftus *et al*., 2005), comprehensive genetic studies can now be undertaken in order to complete the "bigger picture" regarding gene function, such as the linkage of specific gene products to virulence, mating, growth and survival.

3. LITERATURE CITED

Casadevall A., Steenbergen J., Nosanchuk J. (2003). 'Ready made' virulence and 'dual use' virulence factors in pathogenic environment-the *Cryptococcus neoformans* paradigm. *Curr. Opin. Microbiol.* **6:** 332-337.

Chuck S. L., and Sande M. A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **321:** 794–799.

Franzot S. P., Fries B. C., Cleare W., Casadevall A. (1998). Genetic Relationship between *Cryptococcus neoformans* var. *neoformans* Strains of Serotypes A and D. *J. Clin. Microbiol*. **36, 8:** 2200-2204.

Halliday C., Bui T., Krockenberger M., Malik R., Ellis D., Carter D. (1999). Presence of α and *a* mating types in environmental and clinical isolates of *Cryptococcus neoformans* var. *gatti* strains from Australia. *J. Clin. Microbiol.* **37:** 2920-2926.

Lazéra M. S., Pires F. D., Camillo-Coura L., Nishikawa M. M., Bezerra C. C., Trilles L., Wanke B. (1996). Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. *J. Med. Vet. Mycol.* **34, 2:** 127-31.

Loftus B. J., Fung E., Roncaglia P., Rowley D., Amedeo P., Bruno D., Vamathevan J., Miranda M., Anderson I. J., Fraser J. A., Allen J. E., Bosdet I. E., Brent M. R., Chiu R., Doering T. L., Donlin M. J., D'Souza C. A., Fox D. S., Grinberg V., Fu J., Fukushima M., Haas B. J., Huang J. C., Janbon G., Jones S. J., Koo H. L., Krzywinski M. I., Kwon-Chung J. K., Lengeler K. B., Maiti R., Marra M. A., Marra R. E., Mathewson C. A., Mitchell T. G., Pertea M., Riggs F. R., Salzberg S. L., Schein J. E., Shvartsbeyn A., Shin H., Shumway M., Specht C. A., Suh B. B., Tenney A., Utterback T. R., Wickes B L., Wortman J R., Wye N. H., Kronstad J. W., Lodge J. K., Heitman J., Davis R. W., Fraser C. M., Hyman R. W. (2005). The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans. Science.* **307:** 1321-1324.

Meyer W., Marszewska K., Amimostofian M., Igreja R. P., Hardtke C., Methling K., Viviani M. A., Chindamporn A., Sukroongreung S., John M. A., Ellis D. H., Sorrell T. C. (1999). Molecular typing of global isolates of *Cryptococcus neoformans*

var. *neoformans* by polymerase chain reaction fingerprinting and randomly amplified polymorphic DNA - a pilot study to standardize techniques on which to base a detailed epidemiological survey. *Electrophoresis.* **20:** 1790-1799.

Min K. L., Kim Y. H., Kim Y. W., Jung H. S., Hah Y. C. (2001). Characterization of a Novel Laccase Produced by the Wood-Rotting Fungus *Phellinus ribis. Arch. Biochem. Biophys.* **392, 2:** 279-286.

Mitchell T. G. and Perfect J. R. (1995). Cryptococcosis in the Era of AIDS—100 Years after the Discovery of *Cryptococcus neoformans*. *Clin. Microbiol. Rev.* **8, 4:** 515-548.

Randhawa H. S., Mussa A. Y., Khan Z. U. (2000). Decaying wood in tree trunk hollows as a natural substrate for *Cryptococcus neoformans* and other years-like fungi of clinical importance. *Mycopathologia.* **151:** 63-69.

Sorrell T., Ellis D. (1997). Ecology of *Cryptococcus neoformans*. *Rev. Iberoam. Microbiol.* **14:** 42-43.

Trilles L., Lazéra M., Wanke B., Theelen B., Boekhout T. (2003). Genetic characterization of environmental isolates of the *Cryptococcus neoformans* species complex from Brazil. *Med. Mycol.* **41, 5:** 383-390.

APPENDICES

Appendix A

Table 1 Environmental and clinical strains of *Cryptococcus neoformans* isolated throughout the Gauteng, North West and Western Cape Provinces of South Africa.

Reference Number	Origin of Isolation of Strain	HIV Status of Patient
ABO[1]-C13	Soweto, JHB[4], Gauteng	N/I
ABO-C14	Soweto, JHB, Gauteng	Positive
ABO-CF	Western Cape	N/I
CBS[2] 10571	Soil, Wolmaranstad, North West Province	N/A[6]
CBS 10572	Soil, Wolmaranstad, North West Province	N/A
CBS 10573	Soil, Wolmaranstad, North West Province	N/A
CBS 10574	Soil, Wolmaranstad, North West Province	N/A
CBS 10075	Western Cape	N/I
MRC[3] 8855	N/I[5]	N/I
MRC 8856	Robertson, Western Cape	Positive
MRC 8857	N/I	N/I
MRC 8858	Hout Bay, Western Cape	Positive
MRC 8859	Wynberg, Western Cape	Positive
MRC 8860	Wynberg, Western Cape	Positive
MRC 8861	N/I	Positive
MRC 8862	Retreat, Western Cape	Positive
MRC 8863	Langa, Western Cape	Positive
MRC 8864	Masiphumelele, Western Cape	Positive
MRC 8865	Rondebosch, Western Cape	Positive
MRC 8866	Athlone,Western Cape	Positive
MRC 8867	Wetlands, Western Cape	Positive
MRC 8868	Hout Bay, Western Cape	Positive
MRC 8878	Western Cape	N/I
MRC 8879	Western Cape	N/I

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Table 1 Cont. Environmental and clinical strains of *Cryptococcus neoformans* isolated throughout the Gauteng, North West and Western Cape Provinces of South Africa.

Reference Number	**Origin of Isolation of Strains**	**HIV status of Patient**
MRC[3] 8880	Western Cape	N/I[5]
MRC 8882	Western Cape	N/I
MRC 8883	Western Cape	N/I
MRC 8884	Western Cape	N/I
MRC 8885	Western Cape	N/I
MRC 8887	Midrand Ivory Park, JHB[4], Gauteng	Positive
MRC 8888	Thembisa, JHB, Gauteng	Positive
MRC 8889	Tshepisong; Roodeport; Gauteng	N/I
MRC 8890	Khutsong, Carletonville; Gauteng	N/I
MRC 8891	Kagiso; Krugersdorp; Gauteng	Positive

[3]Medical Research Council, PROMEC Unit, Tygerberg, Western Cape, South Africa; [4]Johannesburg (JHB); [5]Not Indicated (N/I)

Appendix B

Table 1 Various environmental sources sampled throughout South Africa and screened for the presence of *Cryptococcus neoformans* and *C. gattii* on Niger Seed agar.

Province Sampled	**Sample Description**	**Number of Samples Collected at Site**
Eastern Cape	Knysna forest soil	10
Eastern Cape	George soil	2
Eastern Cape	Oudtshoorn soil	2
Free State	Bloemfontein soil	5
Gauteng	Bottle brush leaves	2
Gauteng	Bottle brush flowers	2
Gauteng	Bottle brush bark	2
Gauteng	Bottle brush soil	2
Gauteng	Jacaranda leaves	2
Gauteng	Jacaranda flowers	2
Gauteng	Jacaranda bark	2
Gauteng	Jacaranda soil	2
Gauteng	Tree leaves	2
Gauteng	Trees flowers	2
Gauteng	Tree bark	2
Gauteng	Tree soil	2
Kwa-Zulu Natal	Umhlanga soil mix	1
Kwa-Zulu Natal	Umloti soil mix	2
Mpumalanga	*Eucalyptus* soil	4
Mpumalanga	*Eucalyptus* debris	5
Mpumalanga	*Eucalyptus* bark	2
Mpumalanga	*Eucalyptus* leaves	1
Mpumalanga	Tree bark	1
Mpumalanga	Tree sand	1
North West	Wolmaranstad soil	12
North West	Wolmaranstad *Eucalyptus* sp	3

Table 1 Cont. Various environmental sources sampled throughout South Africa and screened for the presence of *Cryptococcus neoformans* and *C. gattii* on Niger Seed agar.

Province Sampled	**Sample Description**	**Number of Samples Collected at Site**
Northern Cape	*Eucalyptus* soil	6
Transkei	*Eucalyptus* soil	8
Western Cape	*Eucalyptus* leaves	4
Western Cape	*Eucalyptus* bark	3
Western Cape	*Eucalyptus* flowers	4
Western Cape	*Eucalyptus* soil	2
Western Cape	Jasmine leaves	1
Western Cape	Jasmine flowers	1
Western Cape	Silver tree leaves	1
Western Cape	Silver tree flowers	1
Western Cape	Seaweed	7
Western Cape	Rotting wood	3
Western Cape	Tokai rotting wood	5
Western Cape	Kirstenbosch rotting wood	1
Western Cape	Brede river soil	4
Western Cape	Apple orchid soil	4
Western Cape	Tygerberg soil	2
Western Cape	Cultivated soil	6
Western Cape	JS Marias soil	2
Western Cape	Uncultivated soil	2
Western Cape	Stellenbosch Botanical Garden soil	4
Western Cape	Western Cape soil	90
Western Cape	Avian Guano	20
Western Cape	Soil containing avian guano	10
Western Cape	Soil containing chicken guano	2

Appendix C

Analysis of Internal Transcribed Spacer Region

Sequencing of the 5S ribosomal RNA, 17S ribosomal RNA genes, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 25S ribosomal RNA gene for *C. neoformans* and *C. gattii* strains obtained during the current study

C. neoformans var. *grubii* ABO-C13

GCTCTGAGCGAGTGAGGAGGTCAGTAGAGAATATTGGACTTCGGTCCATTTATCTACCCAT
CTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAAACTTCTAAATGTAATGA
ATGTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCACATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTCATG
AAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGCGAC
CTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCAGCCC
GGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCATCTC
TTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCAATAAGGCG
GAG

C. neoformans var. *grubii* ABO-C14

AAACGCTCTGAGCGAGTGAGGAGGTCAGTAGAGAATATTGGACTTCGGTCCATTTATCTA
CCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAAACTTCTAAATGT
AATGAATGTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCA
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAG
TCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCG
CGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCA
GCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCA
TCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCAATAA
GGCGGAGGAA

C. neoformans var. *grubii* ABO-CF

GACTTCTGTGATTTAGTCTCACTGCGTGAGCGGACGAAAACAAAAACACCTAAAATGTGG
AATATAGCATATAGTCGACAAGAGAAATCTACGAAAAACAAACAAAACTTTCAACAACG
GATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTG
CAGCCATCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCCTCGGCATTCCGGGGGGC
ATGCCTGTTTGAGCGTCGTTTCCATCTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGCGGA
CGACGTGTAAAGAGCGTCGGAGCTGCGACTCGCCTGAAAGGGAGCGAAGCTGGCCGAGC

GAACTAGACTTTTTTTCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGCGAGACAACAAA
AAGCTCGACCTCAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAG
GAA

C. neoformans var. *grubii* CBS 10571

CGTAGTGACTGCGGAGGATCAGTAGAGAATATTGGACTTCGGTCCATTTATCTACCCATCT
ACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAAACTTCTAAATGTAATGAAT
GTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCACATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT
CTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTCATGAA
AATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGCGACCTG
CAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCAGCCCGGC
GTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCATCTCTTT
TTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCATTAAGCGGAG
GAA

C. neoformans var. *grubii* CBS 10572

TCGTAGTGACCTGCGGAAGGATCATTACAGTATTCTTTTGCCAGCGCTTAACTGCGCGGCG
AAAAACCTTACACACAGTGTCTTTTTGATACAGAACTCTTGCTTTGGTTTGGCCTAGAGAT
AGGTTGGGCCAGAGGTTTAACAAAACACAATTTAATTATTTTTACAGTTAGTCAAATTTTG
AATTAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAG
CGAAATGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATCTTTGAACGCAC
ATTGCGCCCTCTGGTATTCCAGAGGGCATGCCTGTTTGAGCGTCATTTCTCTCTCAAACCC
CCGGGTTTGGTATTGAGTGATACTCTTAGTCGGACTAGGCGTTTGCTTGAAAAGTATTGGC
ATGGGTAGTACTGGATAGTGCTGTCGACCTCTCAATGTATTAGGTTTATCCAACTCGTTGA
ATGGTGTGGCGGGATATTTCTGGTATTGTTGGCCCGGCCTTACAACAACCAAACAAGTTTG
ACCTCAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCATTAAAGCGGAAGGAAA

C. neoformans var. *grubii* CBS 10573

CGGTAAGTGACCTGCGGAAGGATCAGTAGTAGAATATTGGACTTCGGTCCATTTATCTACC
CATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAGACTTCTAAATGTAA
TGAATGTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCACA
TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTC
ATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGC
GACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCAG

CCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCAT
CTCTTTGTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCAATAAG
CGGAGGAAAAAAAGAG

C. neoformans var. *grubii* CBS 10574

CTCCGTAGTGACTGCGGAGGACAGTAGAGAATATTGGACTTCGGTCCATTTATCTACCCAT
CTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAAACTTCTAAATGTAATGA
ATGTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCACATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTCATG
AAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGCGAC
CTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCAGCCC
GGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCATCTC
TTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCATTTAAAGC
GGGAGGAAAA

C. neoformans var. *gattii* CBS 10575

AGCATTCTGGGATTTAGTCTACACTGGCGTGAGCGGCACGAAAACAAAAACACCTAAAAT
GGTGGAATATAGCATATAGTCGACAAGAGAAATCTACGAAAAACAAACAAAACTTTCAA
CAACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTG
AATTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCCTCGGCTTCCGGG
GGGTATGCCTGTTCCCCTTTTTTCCATCTTGCCCCAGTCGCAGATCGATGGCTCCCCGGTCA
TCGTACGTCCTGTCGCCACTTCTCCCCGTAGATTCGCCCCTCTCCCTC

C. neoformans var. *grubii* MRC 8855

CGTTCGCGTCCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTT
TACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCAACA
ACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAA
GGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGAC
TTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTG
GGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTC
GGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTG
AACTTAAGCATATCAAAAGCCGGGAGGAA

***C. neoformans* var. *neoformans* MRC 8856**

ATTTGAGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTA
CACAAACTTCTAAATGTAATGAATGTAATCATATTATAACAATAATAAAACTTTCAACAAC
GGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCAGTGAATCATCGAGTCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGG
GCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTT
GGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGG
AAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGG
CTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAA
CTTAAGCATATCAAAA

***C. neoformans* var. *grubii* MRC 8857**

AATTCGTGTCCTTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTT
ACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCAACAA
CGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAG
GGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACT
TGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGG
GAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCG
GCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGA
ACTTAAGCATATCAAAAGCCGGGAGGAAG

***C. neoformans* var. *grubii* MRC 8858**

ACGTTCGAGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTT
TACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCAACA
ACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAA
GGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGAC
TTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTG
GGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTC
GGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTG
AACTTAAGCATATCAAAAAGCGGGAGGAAA

***C. neoformans* var. *grubii* MRC 8859**

GGACATTCGGGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACG
TTTTACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCA
ACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT

GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCC
GAAGGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTG
GACTTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTA
GTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGT
CTTCGGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCC
GCTGAACTTAAGCATATCAATAAGCGGAGGAAAAA

C. neoformans var. *grubii* MRC 8860

GGAACATTCGGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACG
TTTTACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCA
ACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCC
GAAGGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTG
GACTTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTA
GTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGT
CTTCGGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCC
GCTGAACTTAAGCATATCAAAAGCGGAGGAA

C. neoformans var. *grubii* MRC 8861

GGACGTTCGAGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACG
TTTTACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCA
ACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCC
GAAGGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTG
GACTTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTA
GTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGT
CTTCGGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCC
GCTGAACTTAAGCATATCTA

C. neoformans var. *grubii* MRC 8862

TGAGCACTTCGGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACG
TTTTACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCA
ACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCC
GAAGGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTG
GACTTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTA
GTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGT

CTTCGGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCC
GCTGAACTTAAGCATATCAAA

C. neoformans var. *grubii* MRC 8863

AGTTCGGGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTT
ACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCAACAA
CGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAG
GGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACT
TGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGG
GAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCG
GCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGA
ACTTAAGCATATCAATAAGGCGGAGGGAAAA

C. neoformans var. *grubii* MRC 8864

CATTCGAGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTT
ACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCAACAA
CGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAG
GGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACT
TGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGG
GAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCG
GCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGA
ACTTAAGCATATCAA

C. neoformans var. *grubii* MRC 8865

CTTCGAGTCCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTT
ACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCAACAA
CGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAG
GGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACT
TGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGG
GAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCG
GCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGA
ACTTAAGCATATCAAA

***C. neoformans* var. *grubii* MRC 8866**

GTAATTTGGACTTCGGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGG
CACGTTTTACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACT
TTCAACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTA
ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTA
TTCCGAAGGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCT
GTTGGACTTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGT
GTTAGTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGG
TAGTCTTCGGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCT
ACCCGCTGAACTTAAGCATATCATAAAGCCGGAGGAA

***C. neoformans* var. *grubii* MRC 8867**

GGACGTTCGGGTCCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCAC
GTTTTACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTC
AACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTC
CGAAGGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTT
GGACTTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTT
AGTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAG
TCTTCGGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCC
GCTGAATTTAAGCATATCAATAAGCCGGAGGAAA

***C. neoformans* var. *grubii* MRC 8868**

GGACTTCGGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTT
TACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAAACTTTCAACA
ACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAA
GGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGAC
TTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTG
GGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTC
GGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTG
AACTTAAGCATATCAAAAA

***C. neoformans* var. *grubii* MRC 8878**

GACTTCTGTGATTTAGTCTCACTGCGTGAGCGGACGAAAACAAAAACACCTAAAATGTGG
AATATAGCATATAGTCGACAAGAGAAATCTACGAAAAACAAACAAAACTTTCAACAACG
GATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTG

CAGCCATCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCCTCGGCATTCCGGGGGGC
ATGCCTGTTTGAGCGTCGTTTCCATCTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGCGGA
CGACGTGTAAAGAGCGTCGGAGCTGCGACTCGCCTGAAAGGGAGCGAAGCTGGCCGAGC
GAACTAGACTTTTTTTCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGCGAGACAACAAA
AAGCTCGACCTCAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAG
GAA

C. neoformans var. *grubii* MRC 8879

TTCTGTGATTTAGTCTCACTGCGTGAGCGGACGAAAACAAAAACACCTAAAATGTGGAAT
ATAGCATATAGTCGACAAGAGAAATCTACGAAAAACAAACAAAACTTTCAACAACGGAT
CTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTGCAG
CCATCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCCTCGGCATTCCGGGGGGCATG
CCTGTTTGAGCGTCGTTTCCATCTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGCGGACGA
CGTGTAAAGAGCGTCGGAGCTGCGACTCGCCTGAAAGGGAGCGAAGCTGGCCGAGCGAA
CTAGACTTTTTTTCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGCGAGACAACAAAAAG
CTCGACCTCAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCATAAGCGGAGGAA

C. neoformans var. *grubii* MRC 8880

TTCTGTGATTTAGTCTCACTGCGTGAGCGGACGAAACAAAAACACCTAAAATGTGGAATA
TAGCATATAGTCGACAAGAGAAATCTACGAAAAACAAACAAAACTTTCAACAACGGATCT
CTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTGCAGCC
ATCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCCTCGGCATTCCGGGGGGCATGCC
TGTTTGAGCGTCGTTTCCATCTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGCGGACGACG
TGTAAAGAGCGTCGGAGCTGCGACTCGCCTGAAAGGGAGCGAAGCTGGCCGAGCGAACT
AGACTTTTTTTCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGCGAGACAACAAAAAGCT
CGACCTCAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

C. neoformans var. *grubii* MRC 8882

CCGTAGATTTGGACTTCGGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCTT
CGGCACGTTTTACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAAA
ACTTTCAACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTTG
GTATTCCGAAGGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATTA
CCTGTTGGACTTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAAA
TGTGTTAGTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATGG
GGTAGTCTTCGGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGGG
CTACCCGCTGAACTTAAGCATATCTA

***C. neoformans* var. *grubii* MRC 8883**

CAAATTCTGTGATTTAGTCTACACTGCGTGAGCGGTACGAAAACAAAAACACCTAAAATG
TGGAATATAGCATATAGTCGACAAGAGAAATCTACGAAAAACAAACAAAACTTTCAACA
ACGGATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAA
TTGCAGCCATCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCCTCGGCATTCCGGGG
GGCATGCCTGTTTGAGCGTCGTTTCCATCTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGC
GGACGACGTGTAAAGAGCGTCGGACTGCGACTCGCCTGAAAGGGAGCGAAGCTGGCCAC
GAACTAGACTTTTTTTCAGGGACGCTTGCCGGCGGGAGAGCGAGTGTTGCTAGACTCAAA
AAGCTCGACCTCAAATCAGGTAGGATTTACCCGCTGAAAGTATACGGTATCAGTAAGCGG
AGGGATACGGGTGTGTCCGTTTCGCATAGCGAGTC

***C. neoformans* var. *grubii* MRC 8884**

GACATCATAGCGGAGGAATCCTGCGTGAGCGGACGAAAACAAAAACACCTAAAATGTGG
AATATAGCATATAGTCGACAAGAGAAATCTACGAAAAACAAACAAAACTTTCAACAACG
GATCTCTTGGTTCTCGCATCGATGAAGAGCGCAGCGAAATGCGATACCTAGTGTGAATTG
CAGCCATCGTGAATCATCGAGTTCTTGAACGCACATTGCGCCCCTCGGCATTCCGGGGGGC
ATGCCTGTTTGAGCGTCGTTTCCATCTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGCGGA
CGACGTGTAAAGAGCGTCGGAGCTGCGACTCGCCTGAAAGGGAGCGAAGCTGGCCGAGC
GAACTAGACTTTTTTTCAGGGACGCTTGGCGGCCGAGAGCGAGTGTTGCGAGACAACAAA
AAGCTCGACCTCAAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAG
GAA

***C. neoformans* var. *grubii* MRC 8885**

GCTGTAGATTTGGACTTCGGTCATTTATCTACCCATCTACACCTGTGAACTGTTTATGTGCT
TCGGCACGTTTTACACAAACTTCTAAATGTAATGAATGTAATCTTATTATAACAATAATAA
AACTTTCAACAACGGATCTCTTGGCTTCCACATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACTTGCGCCCTTT
GGTATTCCGAAGGGCATGCCTGTTTGAGAGTCATGAAAATCTCAATCCCTCGGGTTTTATT
ACCTGTTGGACTTGGATTTGGGTGTTTGCCGCGACCTGCAAAGGACGTCGGCTCGCCTTAA
ATGTGTTAGTGGGAAGGTGATTACCTGTCAGCCCGGCGTAATAAGTTTCGCTGGGCCTATG
GGGTAGTCTTCGGCTTGCTGATAACAACCATCTCTTTTTGTTTGACCTCAAATCAGGTAGG
GCTACCCGCTGAACTTAAGCATATCATA

***C. neoformans* var. *grubii* MRC 8887**

CCTTACGAGGACTGCGGAGGACAGTAGAGAATATTGGACTTCGGTCCATTTATCTACCCAT
CTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAAACTTCTAAATGTAATGA
ATGTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCACATCG

ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTCATG
AAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGCGAC
CTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCAGCCC
GGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCATCTC
TTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCAAAAGGCGG
AGG

C. neoformans var. *grubii* MRC 8888

CCCTTACGAGGACTGCGGAGGACAGTAGAGAATATTGGACTTCGGTCCATTTATCTACCC
ATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAAACTTCTAAATGTAAT
GAATGTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCACAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC
GAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTCA
TGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGCG
ACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCAGC
CCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCATC
TCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCAAAAGGC
GGAG

C. neoformans var. *grubii* MRC 8889

TTCCCTTACGAGGACTGCGGAGGACAGTAGAGAATATTGGACTTCGGTCCATTTATCTACC
CATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAAACTTCTAAATGTAA
TGAATGTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCACA
TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTC
ATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGC
GACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCAG
CCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCAT
CTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCAAAAGG
CGGAGGAAA

C. neoformans var. *grubii* MRC 8890

TACACCGAGAGGACTGCGGAGGACAGTAGAGAATATTGGACTTCGGTCCATTTATCTACC
CATCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAAACTTCTAAATGTAA
TGAATGTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCACA
TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTC

ATGAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGC
GACCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCAG
CCCGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCAT
CTCTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCAATAAG
CGGAGGAAATA

C. neoformans var. *grubii* MRC 8891

AATCAAGAGGACTGCGGAGGWCAGTAGAGAATATTGGACTTCGGTCCATTTATCTACCCA
TCTACACCTGTGAACTGTTTATGTGCTTCGGCACGTTTTACACAAACTTCTAAATGTAATG
AATGTAATCTTATTATAACAATAATAAAACTTTCAACAACGGATCTCTTGGCTTCCACATC
GATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCG
AATCTTTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGAGTCAT
GAAAATCTCAATCCCTCGGGTTTTATTACCTGTTGGACTTGGATTTGGGTGTTTGCCGCGA
CCTGCAAAGGACGTCGGCTCGCCTTAAATGTGTTAGTGGGAAGGTGATTACCTGTCAGCC
CGGCGTAATAAGTTTCGCTGGGCCTATGGGGTAGTCTTCGGCTTGCTGATAACAACCATCT
CTTTTTGTTTGACCTCAAATCAGGTAGGGCTACCCGCTGAACTTAAGCATATCAAAAGCCG
GAGGAAA

Appendix D

Analysis of 16S ribosomal RNA Region

Sequencing of the 16S ribosomal RNA (rRNA) genes, partial sequence during the current study

Pseudomonas flourescens

CGATCGGAGCCTACCATGCAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTGGCGAGCG
GCGGACGGGTGAGTAATGCCTAGGAATCTGCCTAGTAGTGGGGGATAACGTCCGGAAACG
GGCGCTAATACCGCATACGTCCTACGGGGGAAAGTGGGGGATCTTCGGACCTCACGCTAT
TAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATC
CGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGC
GTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACC
TAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAG
CCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG
GTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACT
GGCAAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGA
TATAGGAAGGAACACCAGTGGCGAAGCGACCACCTGGACTGATACTGACACTGAGTGCG
AAAGCGTGGGAGCAAACAGATTAGATACCCTGGTAGTCCACGCCGTAACGATGTCACTAG
CCGTTGGGAGCCTTGAGCTCTATGCGCAGCTAACGCATAAGTTGACCGCCTGGGGAGTAC
GCGCAGTAAACTCAATGAATTGACGGACCGCCAAGCGGTGACATGGGTTATCAGCACGGA
ACTTACAGCCTGATCATGAACTTTCTAAAAAAATTGGGGCTCTT

***Enterobacter* sp.**

CGTACGCTGCTAACATGCAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGA
GTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGA
AACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTAGGGCCTCTTG
CCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGAC
GATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGC
CGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGATAA
GGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCA
GCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAC
GCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGA
AACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG
TAGAGATCTGGAGGAATACCGGTGGCGAAGCGGCCCCCTGGACAAAGACTGACGCTCAGT
GCGAAAGCGTGGGAGCAACAGGATTAGATACCCTGTAGTCACGCCGTAACGATGTCGACT

TGAGGTGTGCCCTGAGCTGCTCAGAGCTACGCGTAAGTCGACGCTGGGAGTACGCGCAGT
AAACTCAATGATGACGGGCCGCAAGCGGTGACCTGGATATCAGCACCGAGACCTTACCAC
TTGACTCGAGAACTTCCAGAAA